A STUDY ON DETECTION AND CHARACTERISATION OF METALLO BETALACTAMASE PRODUCING PSEUDOMONAS AERUGINOSA

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BONAFIDE CERTIFICATE

This is to certify that the dissertation entitled **"A STUDY ON DETECTION AND CHARACTERISATION OF METALLO BETA LACTAMASE PRODUCING PSEUDOMONAS AERUGINOSA"** submitted by **Dr.B.DIVYA** to the Tamilnadu Dr. M.G.R Medical University, Chennai, in partial fulfillment of the requirement for the award of M.D. Degree Branch – IV (Microbiology) is a bonafide research work carried out by her under direct supervision & guidance.

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DECLARATION

I, **Dr.B.DIVYA** declare that, I carried out this work on **"A STUDY ON DETECTION AND CHARACTERISATION OF METALLO BETALACTAMASE PRODUCING PSEUDOMONAS AERUGINOSA"** at the institute of Microbiology, Madurai Medical College, I also declare that this bonafide work or a part of this work was not submitted by me or any others for any award, degree, or diploma to any other University, Board, either in India or abroad.

This is submitted to the Tamilnadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the rules and regulations for the M.D Degree examination in Microbiology.

Place: Madurai **Dr. B.DIVYA**
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CONTENTS

INTRODUCTION

Pseudomonas aeruginosa has become one of the most dreadful causes of nosocomial bacterial infections especially in the lung, blood and urinary tract. As a result of its considerable potential to become resistant to many antibiotics, more multidrug resistant strains are encountered as clinical isolates, leaving physicians with a decreasing armamentarium of effective drugs for treatment.

Before the advent of modern medical microbiology, there was evidence that ***P.aeruginosa*** was a cause of serious wound and surgical infections, as elaborated by **Doggett** .In 1850, it was noted by **Sedillot** that there were sometimes blue green discharges on surgical dressings that were associated with infection. In 1862**, Luke** noted rod-shaped microscopic entities within the blue green pus. In 1882, **Gessard** isolated the organisms and originally designated them as bacillus pyocyaneus, and other early Microbiologists also isolated the organism from infected sites. **Osler** in 1925, thought that the organism to be more of a secondary opportunistic invader of damaged tissues as opposed to a primary cause of infection in healthy tissues.

P. aeruginosa has emerged as a major human pathogen in the 1960's, since it can cause infection in immunosuppressed and burns patients as well as cystic fibrosis patients, all of whom were surviving much longer with advanced medical treatments.

Community acquired *P.aeruginosa* infection is most commonly associated with exposure to moist environments. *P.aeruginosa* skin infections which are related to use of whirl pools, hot tubs swimming pools and other types of baths are well recognized clinical presentation of community-acquired infection.

P.aeruginosa nosocomial infections are usually acquired in the hospital, particularly in patients on mechanical ventilation, antibiotic therapy, chemotherapy (or) postsurgery. Endogenous *P.aeruginosa* brought into ICUs by patients from the community can serve as sources of serious infection. With increasing problems due to infection with *P.aeruginosa* strains which are resistant to multiple antibiotics due to inherent and acquired resistance, therapeutic problems with this organisim will remain an imporant cause of morbidity and mortality in tertiary care centres. Biofilm formation on implanted medical devices from venous catheters to orthopedic implants favours the organism to colonize and disseminate systemically.

DISCOVERY OF ANTIBIOTICS & ANTIMICROBIAL RESISTANCE

EVOLUTION OF ANTIMICROBIAL RESISTANCE IN PSEUDOMONAS

Discovery of antimicrobials is one of the most important developments in modern medicine. The modern era of chemotherapy began in 1935 after the discovery of Sulphonamides by German chemist **Gerhard Domagk** [40] which was marketed by **Bayer** by the trade name **Prontosil**.

Penicillin was discovered by **Alexander fleming** in 1928 [83] and it was therapeutically used by **Howard Florey** in 1940. Streptomycin was discovered by an American microbiologist **Selman Walksman**[22] in 1943. In 1948, Cephalosporin was identified by **Brotzu**[41] and introduced for therapeutic use in 1964.Within a very short period after its discovery 1943,resistance to penicillin was developed by many microorganisms. In the recent years, resistance to Cephalosporins and Carbapenems of the Betalactam group have emerged in bacteria leaving behind a very few antibiotics in the tunnel for treatment of the multidrug resistant organisms which pose serious therapeutic concern.

ANTIBIOTIC RESISTANCE:

The mechanisms of bacterial resistance are complex and varied [59].Antimicrobial resistance could be biological Vs clinical, environmentally mediated or microorganism mediated. During the past twenty five years, alarming number of bacterial strains have evolved with resistance to antimicrobial agents[75] .This resistant microbial strains have become one of the major concerns of the clinicians, microbiologists and public health officials. Although genes for mediation of bacterial resistance might have existed before the clinical use of antibiotics, selection of newer resistant strains is mainly by the widespread use of antimicrobials. The most commonly used antibiotics in clinical medicine are the beta-lactam antibiotics and the most common mechanism of resistance to these agents is inactivation by Beta-lactamases. The clinical consequences of therapeutic failure and relapse has extended hospital stays, increased morbidity and mortality, and necessitated the use of potentially more toxic and costly drugs which require newer strategies to prevent the spread of resistant strains and thereby limit future resistance.

EMERGENCE OF NEWER BETALACTAMASES

Pseudomonas aeruginosa is known to be one of the most common pathogens causing hospital acquired infections [50]. Carbapenems are the drugs of choice for the treatment of infections caused by multidrug resistant Gram negative bacilli. Heavy pressure of antibiotic usage has lead to the emergence of newer β- lactamases with a wide spectrum of activity.

Among the various β - lactamases, the Carbapenemases, importantly transferrable Metallobetalactamases are the most worried due to their ability to hydrolyze almost all drugs in that class including the Carbapenems. Metallobetalactamases can hydrolyze all Beta lactams except Monobactams and are not inhibited by β-lactamase inhibitors like Clavulanic acid, Tazabactam or Sulbactum. Metallobetalactamase producing strains are usually resistant to Aminoglycosides and Fluoroquinolnes [132]. However they are sensitive to Polymyxins[133].

The first Metallobetalactamase was reported from Bacillus cereus in the 1960s and since then 18 different Metallobetalactamases have been detected in various Gram-negative bacteria. Production of most of these Metallobetalactamases is chromosomally mediated and did not pose any serious threat of dissemination to other bacteria. The

first metallo β lactamase producing *P.aeruginosa* strain which was plasmid mediated was isolated in Japan in 1988[133]. For many years, these MBL producing isolates were restricted to Japan, but now it has disseminated worldwide [24].In India, Metallobetalactamase producing *P.aeruginosa* was first documented in the year **2002** [90].

With the world wide increase in the occurrence and spread of Metallobetalactamases, early detection of these strains is crucial, as this would benefit by timely implementation of preventive measures like strict infection control practices and therapy with alternative antibiotics [92].As such organisms are being difficult to detect , they are posing significant risks, particularly due to unnoticed spread within institutions and their potential to participate in horizontal MBL gene transfer with other organisms in the hospital[92].

The genes encoding for MBL are typically part of an integron and they are either carried on transferable plasmids or as part of the bacterial chromosome [131]. . Recently, the most common transferable MetalloBetaLactamase families include the VIM, IMP, GIM, SPM, and SIM type enzymes which have been detected worldwide primarily in *Pseudomonas aeruginosa* but were also detected in other Gram-negative bacteria, including the non-fermentors like Acinetobacter spp. and members of the family *Enterobacteriaceae*[112].

Two new subgroups of Metallobetalactamases, designated as NDM-1 and DIM-1, were identified recently, in a clinical isolate of *Klebsiella pneumoniae* in India and in *Pseudomonas stutzeri* in Netherlands, respectively. Such enzymes are emerging in many geographical locations and often mediate very high level resistance to all β lactams except Aztreonam.

Recently, there is an emergence of Carbapenem susceptible isolates often carrying **MBL** genes which are **hidden,** whereby the Microbiologists, clinicians and medical fraternities remain unaware of their presence within the institution [20]. These organisms can appear sensitive to Carbapenems though they carry the carbapenemases. To overcome this, new phenotypic method using both Combined disc test and Double disc synergy test on the same agar plate has been developed, that is supposed to be highly sensitive and specific at detecting both Carbapenem sensitive and Carbapenem resistant isolates across worldwide.

Since there are no CLSI guidelines for detection of Metallobetalactamases, different studies have used different methods. Despite PCR being highly accurate and reliable, its accessibility is limited only to reference laboratories [32]. Various non-molecular methods have been studied, all by using the enzyme's zinc

dependence activity. Chelating agents, such as 2-mercaptopropionic acid, EDTA and other thiol compounds are used to inhibit its activity [32].

The various phenotypic methods used to detect MBL are the Combined disc diffusion test, Double disc synergy test, Modified hodge test and E-test. Other less commonly performed tests are Re-modified hodge test, EDTA-Imipenem Microbiological assay, broth microdilution test. The combined disc diffusion test is based on the principle that in the presence of EDTA, MBL enzyme gets inhibited thus increasing the zone diameter of Imipenem when EDTA is added to it than when tested alone. In the Double disc synergy test, Imipenem or Ceftazidime discs are placed 15-20 mm apart from plain filter paper disc and when EDTA solution is added to the plain disc and incubated, the formation of an extended zone of inhibition from Ceftazidime or Imipenem towards EDTA is interpreted as positive. The Modified hodge test is the test for carbapenemase production which is based on the principle that in the presence of an indicator strain *E.coli* ATCC 25922, the test strain if it produces carbapenemase, it will diffuse in the culture medium and the sensitive indicator strain will grow even in the presence of Imipenem in the vicinity of the test strain producing a clover leaf-like indendation. In

the E-Test, the presence of EDTA in the test strip reduces the MIC for Imipenem by ≥ 3 twofold dilutions if the test strain is an MBL producer. The genotypic method to detect and confirm MBL is the PCR which identifies the genes encoding for MBL production.

Hence, the present study is undertaken to study the prevalence of MBL in Pseudomonas in our locality and various methods are compared to evaluate a simple, cost effective and feasible method which shows sensitivity and specificity equivalent to PCR to detect MBL production & thereby reduce the transmission of resistance strains in the community.

AIMS AND OBJECTIVES

- To study the prevalence of MBL production in *Pseudomonas aeruginosa* isolates from patients admitted to various clinical departments in Govt.Rajaji Hospital, Madurai.
- To Identify and confirm MBL production in *Pseudomonas aeruginosa* isolates by phenotypic and genotypic methods.
- To compare various phenotypic methods in the detection of MBL with genotypic method(PCR) and to evaluate a simple, cost effective and sensitive method to detect MBL production at the earliest that can be recommended for routine screening of MBL in Microbiological laboratories & thereby reduce the transmission of resistant strains in the community.

REVIEW OF LITERATURE

Infectious diseases have been inextricably linked with human health, affecting the development and advancement of societies as well as human evolution [88]. Nosocomial infections are infections causing a serious threat to the community and are reported in 5-10 percent of hospital admissions throughout the world. In India, the nosocomial infection rate is alarmingly rising and is estimated to be about 30-35 percent of all the hospital admissions [33].

With the advent of more elaborate surgical procedures and intensive care, combined with the use of broad spectrum antibiotics and immunosuppressive drugs, the highly virulent Gram negative bacteria have increased in importance in causing nosocomial infections [82] and show high resistance to treatment.

P.aeruginosa is opportunist capable of causing infection in immunocompromised patients. In clinical medicine, *P. aeruginosa* has been primarily encountered as a nosocomial pathogen, which reflects its great propensity to grow in a variety of environment with minimal nutritional components [89].It is usually found in water, soil and plants and associated with colonisation of healthy human and animals. Up to 7% of healthy humans are colonised by *P.aeruginosa* in the nasal mucosa, throat

or on the skin and a high rate of 24% carriage rates in the stool are reported [76]. The organism can tolerate temperatures as high as 45°C to 50°C and can grow in distilled water using dissolved CO_2 and residual iron, sulfur, phosphorus, and divalent cations such as carbon which enhance the growth of *P.aeruginosa* even in antiseptic solutions and other liquids.

Within the health care setting, *P.aeruginosa* colonizes moist surfaces of patients' ear, axilla and perineum and is also found in moist inanimate environments including water in sinks, toilets and showers including antiseptic solutions which are used in the wards [87]. Hospital equipments such as respiratory ventilators, cleansing solutions, mops are also sources of *P.aeruginosa* infection.

The primary factor determining the pathogenic potential of *P.aeruginosa* is the immunological status of the human host. One important predisposing factor for community acquired and nosocomial *P.aeruginosa* infection is neutropenia [113]. Disruption in anatomic barrier functions of skin and mucosal surfaces causes invasive infection.

Patients with significant burn wounds are at high risk for *P.aeruginosa*[120]. Burn wounds, other types of wounds such as chronic non-healing ulcer ,diabetic ulcer ,ulcers due to malignancies, use of intravenous or urinay catheters, use of endotracheal tubes are

predisposing factors to acquire infection with *P.aeruginosa*. In burned skin**, stieritz and holder** [127] showed that *P. aeruginosa* grew to high levels after inoculation as few as 10 organisms, after which there was systemic dissemination and lethality. Though the healthy eye is highly resistant to *P.aeruginosa* infection, when the physical integrity of corneal epithelium is lost, it becomes a pathogen [28, 65, 21].The organism can also cause dreadful infections of the eye. Pseudomonas keratitis and endophthalmitis must be approached as medical emergency that can be fatal and threaten permanent loss of vision [28]. Loss of mucosal barrier is also an important factor in invasive disease. Soluble host factors that have been implicated in high level resistance to *P.aeruginosa* infection include complement proteins [141,107] lung surfactants [91] and similar members of collectin family a variety of cytokines and chemokines[48] .

P.aeruginosa is highly pathogenic in humans because of the multitude, diversity and complexity of its virulence factors. The organism has a large genome more than 6 mega bases in size that is highly plastic in terms of ability to incorporate and modify DNA [124]. All major classes of bacterial virulence systems are virtually found in this organism including exotoxins, endotoxins, leukocidin, type III secreted toxins, fimbriae, flagella, neuraminidase, elastase, proteases, exoenzymes, phospolipases, iron binding proteins, exo-polysaccharides

(alginate),bacteriocins, biofilm formation [27] and pigment production such as pyocyanins[62].

Biofilms are formed on implanated medical devices from venous catheters to orthopaedic implants on which the organism can colonize and disseminate systemically. One of the major bacterial factors implicated in pathogenesis is elastase[118]. Iron levels regulate the ability of *P.aeruginosa* to produce exotoxin, one of the bacterial exoproteins that plays an important role in pathogenesis [107,111]. It uses siderophore systems to acquire iron [16]. The two well studied iron acquisition systems in *P.aeruginosa* are involving the pigments pyoverdin and pyochelin. Pyocyanin, the blue phenazine pigment partly responsible for the colour of *P.aeruginosa* on agar plates, is also detectable in the sputum of CF patients, indicating in vivo production. The production of mucoid morphotype is due to the production of large amounts of polysaccharide (alginate) [98], which is ultimately responsible for the poor prognosis and high mortality rates among patients with cystic fibrosis [80].

Recently, small regulatory RNAs have been recognized whose expression is controlled by a repressor protein fur and these regulator RNAs control production of factors known as **quorum sensors** that signal the organism how to respond to its environment.

QUORUM SENSING - VIRULENCE FACTOR PRODUCTION & BIOFILM FORMATION:

The enormous use of implantable devices in modern clinical medicine has led to the formation of structured bacterial communication known as biofilms on these devices and contribute to infection[27] .Using in vitro systems , it has been shown that QS molecule are needed for the production of structured *P.aeruginosa* bio films. These structures are also produced under conditions of flow of nutrient media over a solid surface where the bio film is forming unlikely to be present in lung or bone but present in vascular tissues [136]. When the bacterial counts increase in a tissue, the organisms reach a critical mass that is thought to allow them to communicate effectively with each other through a system of **quorum sensing (QS)** [25]. At critical bacterial masses, low molecular weight mediators of the QS response are synthesized secreted, diffusing through the cells of the bacterial community to influence gene transcription and virulence factor production[115] .Three important interrelated quorum sensing systems are known designated the **las, rhl** and **pseudomonas quinolone system**[30]. The molecular mediators of the quorum sensing are known as autoinducers (AI) .They have a role in regulation of gene transcription and virulence factor production which has been linked with PA01 chromosome in *P.aeruginosa*[45,119].

TAXONOMY:

The genus *Pseudomonas* and some closely related genera, many of which were formerly placed in the genus *Pseudomonas*, make up a group often referred to as the pseudomonads. The Pseudomonads are classified into 5 ribosomal RNA homology groups by **Palleroni**[97] based on rRNA-DNA homology studies. On the other hand, **Gilardi** classified Pseudomonads into 7 major groups based on the phenotypic characters: fluorescent, stutzeri, alcaligenes, pseudomallei, acidovorans, facilis - delafieldi and diminuta[61].

Among the Pseudomonads, *Pseudomonas aeruginosa* is the well characterised and most frequently recovered Pseudomonad from clinical specimens. *P.aeruginosa* infection is especially prevalent among patients with burn wounds, acute leukaemia, cystic fibrosis, organ transplants and intravenous drug addiction [15].

Infections with Pseudomonas usually occur at sites where moisture accumulates such as trachoestomies, indwelling catheters, burn wounds, the external ear (swimmer's ear) and weeping ulcers [3].The exudation of bluish pus with a grape like odour from the production of pyocyanin is a characteristic feature of *Pseudomonas aeruginosa* infection.

Individual cases of endocarditis, brain abscess, meningitis and bone and joint infections spreading through hematogenous route occur with regular

frequency in the literature [15].Puncture wounds through tennis shoes predispose to serious infections. Perinychia is associated with constant exoposure of extremities to water, detergents or mechanical stress. *Pseudomonas aeruginosa* also causes genito-urinary tract and lower respiratory tract infections; the later can be severe and life threatening in immunocompromised hosts. Sporadic *Pseudomonas aeruginosa* infections following ear piercing have also been reported [56].

MULTIDRUG RESISTANCE IN PSEUDOMONAS:

Treatment of *P.aeruginosa* infection poses a therapeutic challenge for both nosocomial and community-acquired infections and selection of an appropriate antibiotic to initiate treatment is essential to optimize the clinical outcome [14]. A relatively narrow spectrum of antimicrobials is effective including the Carboxypenicillins, the Ureidopenicillins, the antipseudomonal Cephalosporin, Monobactams, Carbapenems, Quinolones and Aminoglycosides [35]. *P. aeruginosa* can develop resistance to antibiotics either through the acquisition of resistance genes on extrachromosomal mobile genetic elements (i.e., plasmids) [71] or through mutational mechanisms that alter the expression and or function of chromosomally mediated mechanisms. Both mechanisms for developing drug resistance can severely limit the therapeutic options

available. [68,94]. Although the availability of certain agents like Doripenem and fourth generation Cephalosporin, Cefipime provided the medical community with a certain degree of security, the situation has changed because of the selection strains of *P. aeruginosa* literally occurring worldwide, carrying the multiple resistant determinants that mediate β - lactam multiresistance along with resistance to Fluoroquinolone and Aminoglycoside group of drugs also [31]. Various definitions defining multi-drug resistant (MDR), extensively drug resistant (XDR) and pandrug-resistant bacteria, including *P. aeruginosa* have been recently reported [73].

MECHANISMS OF ANTIBIOTIC RESISTANCE AND BETA LACTAMASES

Bacteria can express more than one mechanism of antibiotic resistance leading to MDR phenotypes or even pandrug resistance. Molecular analysis of *P. aeruginosa* isolates from a nosocomial outbreak revealed the convergence of several strategies for antibiotic resistance .1) Over expression of Amp C chromosomal β - lactamases conferring resistance to multiple β lactams [71]. 2) Mutational porin loss of OPr D porin, coferring resistance to Imipenem[70]. 3) Upregulation of the Mex

XY efflux system[58] which exports Fluoroquinolones, Tetracyclines, Aminoglycosides and antipseudomonal β lactam agents.

P. aeruginosa carries multiple genetically based resistance determinants, which may act independently or in concert with others [71].Among those of greatest concern are the chromosomal β - lactamases.

BETA LACTAMASES - CLASSIFICATION

β – lactamases are classes of enzymes that inactivate β - lactam antibiotics by splitting the amide bond of the β-lactam ring. They are mediated either by chromosomal genes or by transferrable genes located on plasmids and transposons. In addition, β – lactamases genes *bla* frequently reside on the integrons, which often carry multiple resistant determinants.

β – lactamases can be classified according to the amino acid structure into 4 molecular classes, A to D, as first suggested by Ambler [2]. Alternatively, the Bush- Jacoby-Medeiros system classified the enzymes into several functional groups according to the substrate profile and susceptibility to β – lactamase inhibitors such as clavulanic acid [17].Class A, C and D β – lactamases hydrolyze the β-lactam ring through a serine residue at their active site, whereas class B enzymes are

metallo-β-lactamases that use zinc (zn^{2+}) to break the amide bond. Table.1 and Table.2 show the classification of β – lactamases.

Table.1 Ambler molecular classification of β – lactamases

CLASS	ACTIVE SITE	ENZYME TYPE	SUBSTRATES	EXAMPLES
A	SERINE	a)Broad spectrum Penicillinases	Benzyl penicillin Aminopenicillins Carboxy penicillins Ureidopenicillins	PC1 in S.aureus TEM-1, SHV-1in *E.coli, K.pneumoniae* and other gram negative bacteria
		b)ESBL	Narrow spectrum Cephalosporins Substrates of broad spectrum plus oyimino-β-lactams	In enterobacteriaceae: TEM-Derived,SHV-Derived,CTX-M-Derived, PER-1,VEB-1,VEB-2, GES-1, GES-2, IBC-2 in *Pseudomonas aeruginosa*
		c)Carbapenemases	Substrates of Extended spectrum plus Cephamycins & Carbapenems	KPC-1,KPC-2,KPC-3 in *K.pneumoniae*
B	Metallo-β-lactamases	Carbapenemases	Substrates of Extended spectrum plus cephamycins & Carbapenems	IMP,VIM,GIM,SPM, SIM lineages in *P.aeruginosa*, *Acinetobacter* spp.,

C	SERINE	Cephalosporinases	Substrates of Extended spectrum plus cephamycins	Amp C type enzymes in Enterobacteriaceae, *Acinetobacter baumanni*
D	SERINE	Oxacillinases a)Broad spectrum	Aminopenicillins, Ureidopenicillins, Cloxacillin, Methicillin, Oxacillin & some narrow spectrum Cephalosporins	OXA - family in *P.aeruginosa*
		b)Extended spectrum	Substrates of Extended spectrum plus oxyimino-β-lactams and monobactams	OXA derived in *P.aeruginosa*
		Carbapenemases	Substrates of Extended spectrum plus Cephamycins and carbapenems	OXA derived in *Acinetobacter* spp.,

Table.2 Bush-Jacoby–Medeiros Functional Classification scheme for β – lactamases

Group	Enzyme Type	Inhibition by Clavulanate	Molecular class	Examples
1	Cephalosporinases	No	C	*Enterobacter cloacae* p99(C),MIR-1(P)
2a	Penicillinase	Yes	A	*Bacilluscereus, S.aureus*(B)
2b	Broad spectrum	Yes	A	TEM-1(P),TEM 1(B)
2be	Extended spectrum	Yes	A	K.oxytocaK_1(C),TEM3(P)
2br	Inhibitor resistant	Diminished	A	TEM-30(IRT-2)(P)
2c	Carbenicillinase	Yes	A	AER-1(C),PSE-1(P)
2d	Cloxacillinase	Yes	D/A	*Streptomyces caccoi* (C)
2e	Cephalosporinase	Yes	A	OXA-1(P), FEC-1(P)
2f	Carbapenemase	Yes	A	IMI-1(C), NMC-A(C)
3	Carbapenemase	No	B	*Stenotrophomonas maltophila* L_1(C),IMP-1
4	Penicillinase	No	A	*Burkholderia cepacia*,SAR-2(P)

Note: B - both chromosomal and plasmid, C- chromosomal, P-plasmid.

EMERGENCE OF NEWER BETA LACTAMASES – ESBLs, Amp C and MBLs

The first report on ESBLs appeared in 1983[57], followed in 1988 by reports of AmpC β-lactamases [13]. ESBLs were first found in Europe, most commonly in isolates of *klebsiella spp*.,less commonly in *E.coli* [105].The number of enzymes continue to increase[51].These enzymes have been demonstrated in Enterobacteriaceae and more recently in other genera such as *P. aeruginosa* [134]. PER-1 was the first detected and fully characterized ESBL in *P. aeruginosa*. It was identified in 1991 in France from the urine culture of a Turkish citizen (Nordmann & Naas, 1994) which was encoded in chromosome. Later, plasmid mediated PER-1 enzymes were also reported (Nordmann & Guibert, 1998).ESBLs confer resistance to all β lactams with the major exception of Carbapenems, such as Imipenem and are inhibited by Clavulanic acid.

Plasmid-mediated AmpC β-lactamases have been identified in *Klebsiella pneumoniae, Proteus mirabilis, Salmonella spp.* and *E. coli* and were first documented in 1988 [13]. AmpC β - lactamases are cephalosporinases that inhibit Cephamycins as well as other extended-spectrum Cephalosporins and are poorly inhibited by Clavulanic acid.

METALLO-BETA LACTAMASES

Metallo-β-lactamases are class B β lactamases, which requires divalent cations of zinc as cofactors for enzyme activity and are therefore inhibited by metal chelators. They have potent inhibitory activity not only against Carbapenems but also against other β - lactam antibiotics. Carbapenems, especially Meropenem, Imipenem, and Panipenem (available in Japan only) are potent drugs for the treatment of infections due to multidrug resistant *P.aeruginosa*. However, the prevalence of Carbapenem resistant *P.aeruginosa* strains has been increasing worldwide. The IMP and VIM genes are horizontally transferable via plasmids and can rapidly spread to other bacteria and are responsible for MBL production. Thus, MBL- producing *P.aeruginosa* strains have been reported to be important causes of nosocomial infections which are associated with clonal spread.

Watanabe et al., 1991 [133] first reported *pseudomonas aeruginosa* producing MBLs from Japan in 1991 and Yan et al., 2001[137] and Yatsuyangai et al., 2004[139] documented the reports from various parts of world including Asia.

Luzzaroa et al., 2004[72] described the 5 different types of MBLs whose prevalence is increasing rapidly as, **IMP** (Imipenem hydrolyzing

β-lactamase), **VIM** (Verona Integron encoded Metallo-β-lactamase), **SPM** (Sao Paulo Metallo-β-lactamase), **GIM** (German Imipenemase) and **SIM** (Seoul Imipenemase). Senda et al., 1996[121] stated that IMP and VIM are the most predominant MBLs so far. Lagatolla et al., 2004[63] described that new subtypes of IMP and VIM are being reported commonly and recently 11 subtypes of VIM and 21 subtypes of IMP have also been documented. Lauretti et al., 2004[64], Lee et el., 2005[66] pointed out that MBL producing Gram negative bacteria often are resistant to additional classes of drugs and behave as multi-drug resistant bacteria. Nordman et al., 2002[92] stated that the Gram negative bacteria producing acquired MBLs have been reported under standard conditions and are difficult to recognize.

Lagotta et al., 2004[63], Libisch et al., 2004[69], Patzer et al.,2001[100], Poirel et al., 2000[110] documented the reports of MBLs from Europe, Peleg et al.,2004[101] from Australia, Gales et al., [36] from South America and Toleman et al.,2004[129] from North America. Bandoh et al., 1992[11] reported the transferrable MBLs in *Bacteriodes Fragilis*. Yong et al., [140] documented that apart from *P.aeruginosa*, other bacteria like *Serratia, Klebsiella pneumoniae, Enterobacter aerogenes, E.cloacae, Proteus vulgaris, Citrobacter freundii, P.putida, Acinetobacter* and *Alcaligenes xylosoxidans* were also shown to produce MBL. Galleni et al., 2001[37] and

Garau et al.,[38] proposed a standard numbering scheme and identified three subclasses of class B β – lactamases [B 1 to B 3].

Nordman and poirel et al., 2002 [92] and Walsh et al., 2005 [131] described that class B or the metallo - enzymes (MBL) are the most significant Carbepenemases. Over the last decade MBL producing isolates have emerged particularly in Pseudomonas aeruginosa. Bashir et al., 2011[12], Pitout et al., 2007 [108] described that MBL genes have spread from *P.aeruginosa* to Enterobacteriaceae in recent years, and a clinical scenario is developing that could indicate the global spread. Marr et al., 2006 [79] described the fatal outcome when patients with serious MBL producing organisms are treated with antibiotics to which the organism is totally resistant.

Hirakata et al 1998 [47] studied that Carbepenam - resistant Gram negative bacterial infections with *Pseudomonas aeruginosa and Serratia marcescens* have emerged in Japan, and these isolates are commonly producing IMP -1 metallo-β-lactamase. Arakawa et al., 1995 [7] identified the *bla* $_{IMP}$ genes responsible for the IMP -1 production are usually mediated by integrons which are carried by transferrable plasmids. Senda.k., and Y.Arakawa et al., 1996[121] reported the transmissions of the *bla* $_{IMP}$ gene cassette among various Gram-negative rods. Bush.k.1998

emphasized the importance of early recognition of IMP-1 producers are important for infection control, as they demonstrate a wide range of resistance to various broad spectrum β lactam antibiotics. Wood ford.N.et al.,1998[135] reported the world wide spread of several metallo - β-lactamase producing Gram Negative bacteria outside Japan such as United Kingdom, Italy and Singapore. After being primarily detected in *P.aeruginosa*, MBL was found in other Gram negative bacteria also, as described by Anne Marie Qeennan et al., 2007 [5].

Although various MBL detection techniques have been investigated, there are so far no gold standards or reference phenotypic methods for the detection of all transferrable MBLs [77]. Gupta et al., 2006[42] documented a high prevalence of nosocomial infections due to *P.aeruginosa*. According to Navneeth BV et al.,[90].Gupta V, Datta et al 2006[43] ,Jesudason MV et al.,2005[52] , Agarwal VA et al., and Mendiratta DK et al.,2005[84], production of MBL in *P.aeruginosa* are varying from 7% - 65%. In India, Zavascki et al., 2006[143], Variya et al., 2008[130] and Hirakata et al., 1998[47] showed the prevalence of MBL from 8-14%.Behera et al., 2008[9] stated that either MBL genes are not always expressed or resistance may require uptake of Carbapenems. With the emergence of Carbapenem susceptible MBL carrying organisms, the

decision of which isolates to be chosen for phenotypic MBL detection is controversial. Gibb A.P.et al 2002[39] reported an outbreak of IMP-7 producing *P.aeruginosa* and also documented that *bla* $_{VIM}$ have replaced those producing IMP-7 recently.

M.J.Carvalho et al., 2005[19] reported a new VIM-2 type MBL in *P.aeruginosa* in a portuguese hospital. Mazzoriol.A et al., 2005[81] detected a new VIM-11 type MBL in a clinical isolate of *P.aeruginosa* from Italy. Pena et al., 2005[103] studied the occurrence of metallo-β-lactamase VIM-2 in *P.aeruginosa* isolates resistant to carbapenems in central Portugal. Bashir et al., 2011[12] documented 13.42% of *P.aeruginosa* were resistant to Imipenem and 11.66% were found to be producing MBL and also documented that the interval between admission to hospital and isolation of pathogen was longer in patients infected with MBL producers than MBL non-producers.

Elias et al., 2009[29] reported the nosocomial outbreak caused by a *bla* $_{VIM-2}$ positive *P.aeruginosa* in patients of urology department of the University hospital, Wurzburg. Livermore and Woodford et al., 2000[71] proved that the prolonged hospital stay and prolonged use of antibiotics (especially Carbapenems) are the main risk factors for the emergence of the MBL producing Gram negative organisms.

Debasrita Chakraborthy et al.,2010[26] studied the prevalence of MBL in gram negative infections from ICU in Kolkata and reported 31% of MBL producers from COPD patients and 10.5% from hepatic failure, cancer and cardiac disease patients and 21% were suffering from pneumonia. Supriya Upadhyay et al., 2010[128] studied the different beta lactamase classes among clinical *Pseudomonas aeruginosa* and reported 46.6% as MBL producers among Amp C producing isolates.

Ozgumus et al., 2007[95] studied the molecular epidemiology of *Pseudomonas aeruginosa* isolates carrying IMP-1 gene in a university hospital in Turkey. Noyal et al., 2009[93] found that 50% of resistance in Carbapenems among *P.aeruginosa* was due to the production of MBLs. But Jesudason et al., 2005[52] has documented MBL production among 75% of Imipenem resistant Pseudomonas isolates.

Hirakata et al., 2003 [46] inferred from a study conducted in Japan that patients infected with MBL - producing *P.aeruginosa* were treated with multiple antibiotics and more importantly, that infection related deaths due to MBL-producing *P.aeruginosa* were more frequent than deaths caused by *P.aeruginosa* which were not producing MBLs.

Horieh Saderi et al., 2008[49] observed a resistance of 73.44 % to Ceftazidime among the *P.aeruginosa* and 53.2 % of screened isolates

were MBL positive. Another study from South India by Navaneeth et a l., 2002[90] reported 12 % MBL-mediated Imipenem resistance in *P.aeruginosa.* Mihani et al., 2007[86] reported that 19.51% of Imipenem resistant *P.aeruginosa* strains isolated from burned patients were MBL producers.

Peleg et al., 2005 [102] described the MBL related outbreaks due to treatment of infection with Carbapenems caused by **Carbapenem-sensitive MBLs carrying hidden genes** which involve in horizontal MBL gene transfer with other gram negative pathogens.

Yoshichika et al., 2000[142] evaluated a simple test for screening MBL producing Gram Negative bacteria by using various MBL inhibitors including thiol compounds $CuCl_2$, $FeCl_2$,EDTA and thiol compounds like 2-Mercaptopropionic acid, Mercaptoacetic acid, and Mercaptoethanol and tested for IMP-1 inhibition and used Cefazidime resistant strains to confirm the presence of the *bla* $_{IMP}$ gene. Soo -Young kim et al., 2007[125] performed a study to evaluate the double disc tests involving discs containing Meropenem, Imipenem, Ertapenem and Ceftazidime; discs containing high and low concentrations of Tris - EDTA ,discs containing 2-Mercaptopropionic acid [MPA]; and TE discs supplemented with MPA for the detection of MBLs. In their study, they concluded that by using

IPM and TE disc supplemented with 20 µl of 1:320 MPA, the MBL producers can be detected accurately. Arakawa et al., 2000[7], inferred that Ceftazidime discs used along with MPA provided accurate MBL detection because MBL producing organisms were usually highly resistant to CAZ.

Yong et al., 2002[140] used IPM-EDTA disc method, with 750µg of EDTA in combination with IPM discs with a zone difference of $\geq$ 7 mm between IPM alone and along with EDTA as the criterion to detect MBLs. They obtained excellent sensitivity and specificity to detect VIM-2 type and IMP-1 type producing *P.aeruginosa* and *Acinetobacter spp*. In a study conducted by Agrawal et al.,2008[1], Polymyxin B was most effective antibiotic with 0% resistance similar to the reports of Sarkar et al.,2006[117] followed by Imipenem (8.05%) and Ceftazidime (10.35%).In the Studies of Shahid et al.,2004[122] and Pitt et al.,2003[109] Ceftazidime resistance was 20% and 39.6% respectively.

Johann D.D Pitout et al., 2005[54] inferred that Meropenem alone and along with EDTA showed 100% sensitivity and 97% specificity in identifying MBL producing strains of *P.aeruginosa* and gave good results than Imipenem and the MBL E test. They also performed Duplex PCR assay with excellent sensitivity & Specificity for the simultaneous

confirmation of VIM & IMP genes in *Pseudomonas aeruginosa.* Genotypic methods of detection of MBL producing organisms give specific and accurate results, but due to cost constraints, it is of limited use in all laboratories [53].

Clare Franklin et al 2006 [20] performed the phenotypic detection of Carbapenem sensitive MBL producing gram negative bacilli by using double disc synergy test and a combined disc test with Imipenem and 292 µg of EDTA on one agar plate. They also inferred that out of the 84 MBL carrying isolates 51 were sensitive to atleast one carbapenem. Yan et al., 2004 [138] also studied on the detection of Carbapenem sensitive MBL carrying organisms and compared three methods, the double disc, combined disc and the E test.

Walsh et al.,2002 [132] while evaluating the MBL E test inferred that both Mueller-Hinton Agar and Isosensitest media had excellent sensitivity, 97% for Mueller-Hinton agar & 93% for Isosensitest agar. M.J.C. Noyal et al., 2009[93] evaluated simple screening methods for detection of carbapenemases in non-fermentative Gram negative bacilli and inferred that disc synergy test using EDTA as a better method for detecting MBLs than Modified Hodge test. Rizvi et al., 2009[116] also inferred that disc synergy test using EDTA as a better method for

detecting MBLs than Modified Hodge test. Andrade et al., 2003 [4] has reported that resistance to Carbapenems in *P.aeruginosa* has increased to 40%. Kanungo et al., 2006[55] inferred from a study done at a tertiary care centre in Puducherry, India, 10.9% resistance with Carbapenems in *P.aeruginosa* isolates . Mahendralingam et al., 1996[74] attempted plasmid extraction from all Imipenem and Ceftazidime resistant isolates by alkaline lysis method.

Hemalatha et al., 2005[44] found that Ceftazidime - EDTA could pick up additional isolates of MBL producers than than with Imipenem-EDTA. Bashir et al., 2011[12] compared three different methods for identifying MBL producing *P.aeruginosa*; combined disc test, reduction in MIC with Imipenem plus EDTA combination and E-test. In their study 13.42% of the isolates were resistant to Imipenem and 11.66% were found to be MBL producer by combined disc test and showed zone enhancement of $\geq$ 7 mm around both Imipenem- EDTA disc and Ceftazidime - EDTA disc compared to plain discs.

In 1996, a PCR detection assay was published for the detection of Gram negative bacilli producing IMP-1type MBLs by Senda et al., [121] and a PCR typing scheme for detection of integron associated MBLs was published in 2003 by Shibata et al., [123]. Confirmation of MBLs with

PCR is an important step, since EDTA can give false positive results in due to altered Opr D levels as described by Conejo et al 2003 [23]. Pitout et al.,2005 [108],developed a duplex PCR assay with excellent sensitivity and specificity for the simultaneous confirmation of IMP and VIM genes in MBL producing *Pseudomonas aeruginosa* , whereas PCR typing method was proposed by Shibata et al., in 2003 [123] involves sequencing of the amplicons.

The identification of MBL and other Carbapenemases is of prime importance in choosing the most appropriate antibiotic for the treatment of Carbapenem resistant isolates in any health care set up. The severity of *Pseudomonas aeruginosa* infection can be decreased by early and prompt detection and appropriate treatment before the bacteria change to a mucoid phenotype, which is extremely difficult to eradicate if once established.

Common antipseudomonal drugs are Carbenecillin, Ceftazidine, Piperacillin-Tazobactam & Cefoperazone – Sulbactam. The Carbapenems are one of the last resorts for the treatment of serious multi-drug resistant *Pseudomonas aeruginosa* infection. Meropenem, Ertapenem and Imipenem/Cilastin are effective against most ESBL and AmpC producing organisms. Though these novel carbapenems are more effective against Multi Drug Resistant Pseudomonas infections, resistance is still mediated

through OprD deletions and spread of broad-spectrum Carbapenemases and MBLs. In studies conducted by Walsh et al., 2005[135], intravenous Colistin with Rifampin and Imipenem was suggested for the treatment of Carbapenem resistant isolates without MBL production, whereas the combination of Colistin and Rifampicin with or without Tigecycline was suggested for treatment of MBL producing Carbapenem resistant isolates as described by Maragakis LL et al., 2008 [78] and Perez et al., 2007 [105].

The medical community has now started to use drugs like Colistin and Polymyxin B which were once not used decades ago due to their toxicity but are now being considered as **''antimicrobials for the 21st century''** [85]. However some researchers reported emergence of Colistin resistant organisms in their study [6], which necessitates the discovery of newer molecules to treat the patients earlier and prevent the development and dissemination of resistance in the future.

MATERIALS AND METHODS

It is a prospective study conducted in patients admitted to Government Rajaji Hospital, attached to Madurai Medical College, Madurai. The study was conducted between June 2011 & May 2012. Ethical committee clearance has been obtained from the institution and written informed consent received from the patients before collecting the specimens. A total of 580 clinical samples were collected including pus, sputum, urine, blood, ascitic fluid, endotracheal fluid, broncho-alveolar lavage and wound swab from the patients who were admitted to various clinical departments of Govt. Rajaji Hospital, Madurai.

Inclusion Criteria:

- Males and females of all age groups were included
- Patients affected with burn wounds, Patients with non-healing ulcer
- Diabetic patients with ulcers
- Patients with provisional diagnosis of Septicaemia and Pneumonia
- Patients with indwelling urinary catheter
- Patients on ventilatory support for prolonged period in IRCU
- Patients with peritonitis

COLLECTION OF SPECIMENS

- Collection of Blood sample:

  Blood samples were collected by strict aseptic technique. The skin over the vene puncture site approximately 5cm diameter was cleansed thoroughly with 70 % ethanol followed by povidone iodine and allowed to dry at least for one minute before collecting the sample. 5 ml of blood was collected in 50 ml of Brain Heart Infusion Broth (B.H.I) in adults. In the paediatric age group 1 to 2 ml of blood was collected in appropriate quantity of B.H.I.

- Collection of Pus sample:

  Sterile cotton wool swabs were used to collect the sample from infected sites. The swabs were transported in sterile test tubes to the laboratory. Two swabs were collected from patients and subjected to direct smear and culture. Sterile disposable syringe was used to aspirate pus in case of abscesses.

- Collection of Sputum sample:

  The patients were asked to cough deeply before collecting the sputum to avoid mixing of saliva with the sputum. It was collected in the morning before any mouthwash was used and mouth should be rinsed with saline or water just before expectoration. Clean, dry,

sterile wide mouthed, properly labeled, screw capped and leak proof containers were used for sputum collection.

- Collection of Urine sample:

  Patients were instructed for proper collection of urine samples without any contamination. Male patients were asked to retract the prepuce and clean the urethral meatus with saline and to collect the early morning, mid stream urine. Female patients were asked to clean the genitals with soap and water and to dry the area with sterile gauze pad. The urine was collected with the labia held apart. The specimen was collected in a clean, wide mouthed, screw capped and leak proof container and transported to the laboratory without any delay.

- Collection of wound swab:

  In cases of burn wounds and diabetic patients with ulcers, the wound site was cleaned with sterile saline and before application of any topical antibiotic preparation, two swabs were taken with sterile cotton swabs from the edge of the wound with active infection. The swabs were moistened with sterile saline before collecting the specimen to avoid drying of the specimen before processing.

- Collection of Endotracheal aspirate:

  In patients with tracheostomies, who were unable to produce sputum, the endotracheal secretions were collected by aspirating the fluid by suctioning. The specimen was collected in a sterile container and transported immediately to the laboratory

- Collection of Bronchoalveolar – Lavage(BAL):

  Bronchoscopy assisted bronchial washings or aspirates were obtained by instilling a small amount of sterile physiological saline into the bronchial tree and by withdrawing the fluid. The specimen was collected in a sterile container and transported immediately to the laboratory.

- Collection of ascitic fluid:

  The skin over the site of collection was sterilized with 70% alcohol and atleast 10 ml of fluid was aspirated with sterile syringe and needle and collected in a sterile tube or vial and transported to the laboratory.

- The collected specimens were properly labeled with Name, Age, Sex, I.P/ O.P.No. of the patient, Date and Time of collection, Type of sample and Name of the ward and brought to the laboratory and processed immediately.

PROCESSING OF SAMPLES:

Blood: BHI broth containing blood samples were kept in the incubator at 37˚Cfor 18-24hours after which the broth which showed turbidity was subcultured onto the following media using sterile technique.

1. Nutrient Agar
2. MacConkey Agar
3. Blood Agar

The broths which were clear were kept for further incubation and regarded as negative for growth if it appeared clear even after 48 hours of incubation.

Urine: The urine specimens were centrifuged at 500-1000 g for 5 minutes. The sediment was used for wet mount and if bacteria and pus cells were seen direct Gram staining was done and the supernatant was used for inoculating into culture media and incubation was done at 37˚C for 18-24 hours aerobically.

Wound swab: One swab was used for direct Gram staining and the other swab used for inoculating into solid culture media and incubation was done at 37˚C for a period of 18-24 hours aerobically. After this initial processing the swabs were kept in glucose broth and after overnight incubation the glucose broth was observed for turbidity and if the culture

plates showed no growth after overnight incubation a repeat subculture was done from the glucose broth which contained the swabs.

Pus, Sputum, Endotracheal fuid, Ascitic fluid and BAL: The specimens were processed, first by doing direct Gram staining and then inoculating into culture media and incubation was done at 37˚C for a period of 18-24 hours aerobically.

CULTURE IDENTIFICATION:

After overnight incubation, the culture plates were examined for the presence of growth and the organisms were identified by morphology of colonies on solid media, Gram staining, biochemical reactions and other special identification tests.

ISOLATION AND IDENTIFICATION OF PSEUDOMONAS AERUGINOSA:

Identification was mainly based on the Gram staining, colony morphology on Nutrient Agar, MacConkey Agar and Blood Agar, the characteristic odour in culture plates, oxidase test, motility, biochemical reactions and growth at 42˚C.

GRAM STAINING: From a single isolated colony on Nutrient agar plate, the smear was prepared in clean, dry, grease free slide and it was dried in air and fixed by heating. The smear was then flooded with 0.5% Methyl violet and washed with water after 1 minute. Gram's Iodine was

added to the smear and washed with water after 1 minute. Then the smear was decolorized with one or two drops of Acetone and immediately washed with water. The counter stain, 1:10 dilute Carbol fuschin was added to the smear and washed with water after 1 minute. The smear was then dried with blotting paper and viewed under oil immersion objective. Pink colored bacilli arranged in discrete pattern were identified as Gram Negative Bacilli.

COLONY MORPHOLOGY: *Pseudomonas aeruginosa* was identified based on the typical colony morphology in different culture media as follows:

Nutrient agar: Green pigmented, smooth, large, low convex, translucent colonies with earthy, grape like or corn-taco like odour.

MacConkey agar: Pale or colourless, Non-Lactose fermenting colonies with spreading edges.

Blood agar: Beta hemolytic, Greyish white colonies with metallic sheen.

MOTILITY (HANGING DROP METHOD): A clean cover slip was taken and paraffin was applied to all the four corners. A drop of broth culture was placed on the centre of the cover slip with the sterilized inoculating loop. The cavity slide was taken inverted over the cover slip with the drop so that the drop was placed in the centre. The slide was inverted and focused under 10x and the edge of the drop identified. Then,

without changing the field, the focus was shifted to 40x and observed for the motile organisms. Actively motile bacilli were seen which was supportive for the identification of *P.aeruginosa*.

BIOCHEMICAL REACTIONS: With the following tests *Pseudomonas aeruginosa was* identified biochemically.

Oxidase test (Disc method): The oxidase disc was moistened with normal saline and then the colony from nutrient agar was taken with the help of a sterile glass rod and applied over the disc. Deep blue or purple colour in 10 seconds was taken as positive test.

Catalase test (Tube method): 2-3 ml of 3% Hydrogen peroxide was taken in a clean test tube. Few colonies of the test organism were taken from the culture plate with a sterile glass rod and immersed in the Hydrogen peroxide solution. Brisk effervescence within ten seconds was considered as catalase positive.

TSI (Triple sugar iron medium): An isolated colony from the culture plate was taken with a straight wire loop and stabbed into the butt portion of the TSI medium, withdrawn and streaked in a zig-zag manner over the slant portion and incubation was done at 37˚Cfor 18-24 hours. The observation of alkaline change over the butt portion and alkaline change over slant portion was identified as a non-fermentor.

Citrate utilization test: The well isolated colony from the culture plate was taken with a straight wire loop and inoculated into the Simmon's citrate medium and incubation was done at 37˚C for 18-24 hours. The colour change from green to blue colour or the growth of colonies on the streak line was considered as positive citrate utilization test.

Oxidation-fermentation (O/F) test : Two tubes of O.F medium containing glucose were inoculated with the organism isolated from Nutrient agar plate by stabbing 3-4 times half way to the bottom of the tube .One tube was promptly covered with a layer of sterile melted paraffin jelly to a depth of 5-10 mm, leaving the other tube open to the air. Both the tubes were kept in the incubator at 35˚C for 72 hours and examined daily. The formation of yellow colour (acid) in the open tube and green colour (alkaline) in the sealed tube indicated oxidative metabolism of the organism.

Nitrate reduction test: The nitrate medium was inoculated with a loop full of the test organism isolated in pure culture on agar medium and incubation was done at 35˚C for 18-24 hours. At the end of incubation, 1 ml of Nitrate A (alpha - Naphthylamine and 5 N 30% Acetic acid) and Nitrate B (Sulphanilic acid and 5 N 30% Acetic acid) reagent were added in that order. The development of red colour within 30 seconds after adding the reagents indicated a positive nitrate reduction test. If no colour

developed after addition of reagents, zinc dust was added to confirm a true negative reaction.

Growth at 42˚C: Two tubes of Trypticase soy agar (TSA) were inoculated by streaking over the slant portion with a light inoculum by touching a needle to the top of a single 24 hour old colony from nutrient agar plate. Incubation was done at 35˚C for one tube and the other at 42˚C. Good growth at 35˚C and 42˚C after 24 hours of incubation was considered as positive test for *Pseudomonas aeruginosa*.

Arginine dihydrolase test: A well isolated colony of the test organism previously recovered on primary isolation agar was taken and inoculated into two tubes of Moeller Decarboxylase medium, one containing arginine amino acid and the other tube was used as a control tube devoid of arginine. Both tubes were over layed with sterile mineral oil to cover about 1 cm of the surface and incubation was done at 35˚ c for 18-24 hours. Conversion of the control tube to a yellow colour indicated that the organism was viable and reversion of the tube containing the amino acid to a blue purple colour indicated a positive test owing to the formation of amines from the decarboxylation reaction.

Other biochemical tests done are: Indole production, MR/VP test, Mannitol fermentation, Urea hydrolysis, Aesculin hydrolysis were all negative.

DETECTION OF METALLOBETALACTAMASE PRODUCTION IN PSEUDOMONAS AERUGINOSA BY PHENOTYPIC METHODS:

Antibiotic susceptibility testing was performed for all the *Pseudomonas aeruginosa* isolates as per the standard CLSI guidelines for the following antimicrobials by using Kirby- Bauer disc diffusion method.

Piperacillin (75µg), Piperacillin – Tazobactam (100/10µg), Gentamicin (10 µg), Amikacin (30 µg), Norfloxacin (10µg), Ciprofloxacin (5µg), Netilmicin (30µg), Ceftazidime (30µg), Ceftriaxone (30µg), Imipenem(10µg), Meropenem (10µg), Polymyxin B (300U).

Kirby-Bauer's disc diffusion method:

3-5 well isolated colonies were picked up and emulsified in 2 ml of nutrient broth and incubation was done at 37˚C of 2-4 hours. The turbidity of the inoculum was adjusted to 0.5 McFarland standard using Wickham's chart. Using a sterile cotton swab that was soaked with the broth, a lawn culture was made onto the dried surface of Muller-Hinton agar (MHA). Excess broth was expressed by rotating the swab gently against the inner side of the tube. The plates were allowed to dry for 15 minutes and the pre-determined panel of antibiotic discs were dispensed onto the inoculated MHA plate and incubation was done at 37˚C for 18-

24 hours. The zone diameter was recorded and interpretation was done as sensitive, intermediate and resistant as per the CLSI guidelines 2011.

MIC determination by agar dilution method:

Preparation of media:

Mueller- Hinton agar was prepared in tubes and autoclaved. It was then allowed to cool in a 50^{o}C water bath. Serial dilution of the 3 GCs Ceftazidime and Imipenem were prepared in sterile distilled water to give a final concentration ranging from 0.5µg – 2048µg/ml of agar. After adding the 1 ml of appropriate dilution of drug to the 14 ml of medium at 55^{o} C it was mixed well and poured into sterile petridishes. (The media was used immediately otherwise potency of drugs would be affected. Upto 12 different organisms can be inoculated in a single plate).A control plate containing the test medium without the antibiotic was prepared for each series of test.

Inoculum Preparation:

At least 3-5 well isolated colonies of the similar morphology were selected from an agar culture plate. Top of each colony was touched with a loop and then was transferred into a tube containing 4-5ml of broth and incubation was done at 37^{o}C until it reaches 0.5 Mc Farland's standard (usually 2-6 hrs). This results in growth corresponding to 150 million organisms /ml.

Inoculation of test plates:

Plates of various concentrations were divided into required number (9-12 divisions / plate). 10µl of inoculum was put into the appropriate quadrant and incubation was done at 37^{o}C for 16-18 hours. Minimum inhibitory concentration for Imipenem and Ceftazidime was measured as the lowest concentration of drug at which no visible growth occurs for that particular strain.

Screening and confirmation of MBLs:

The *Pseudomonas aeruginosa* isolates which were found to be resistant to Ceftazidime, Imipenem and Meropenem by Kirby - Bauer disc diffusion method were selected and the minimum inhibitory concentration of resistant isolates were determined and subjected to various phenotypic detection methods such as Combined disc diffusion Test, Modified Hodge Test, Double Disc Synergy Test and E Test and confirmed by genotypic method i.e, PCR. ATCC 27853 *Pseudomonas aeruginosa* was used as a negative control.

Common initial steps – Inoculum preparation:

1. 3-5 colonies of the strain to be tested were touched from 24 hour culture plate with a straight wire loop and transferred to sterile peptone water and incubation was done at 37˚C and turbidity adjusted to 0.5 McFarland standard.
2. A lawn culture was made on cation balanced Mueller Hinton agar plate with a sterile cotton swab using the inoculum.(Excess broth was expressed by rotating the swab against the inner side of the suspension tube before inoculation)
3. Inoculum was then allowed to dry for 3-5minutes but not more than 15 minutes before applying the antibiotic disc or E test strip.

Preparation of EDTA solution:

0.5 M EDTA solution was prepared by dissolving 186.1g of disodium EDTA.2H2O in 1000 ml of distilled water and its pH was adjusted to 8.0 by using NaOH. The mixture was then sterilized by autoclaving. EDTA solution was added on Imipenem and Ceftazidime discs to obtain a desired concentration of 750 μg per disc.

Determination of MIC of Imipenem and ceftazidime with EDTA:

1 ml of EDTA solution was added to solution containing 1 ml of Imipenem and Ceftazidime. 2 ml of EDTA solution with the corresponding drug was mixed with 18 ml of Mueller- Hinton agar. Inoculation and incubation of plates were done as described for MIC determination of the individual drugs without EDTA. The highest dilution which inhibited the growth of the organisms was taken as MIC of Imipenem/Ceftazidime plus EDTA combination and was compared to MIC of Imipenem/Ceftazidime alone. A minimum fourfold reduction in MIC with Imipenem/Ceftazidime plus EDTA combination when compared to the MIC of individual drugs alone was interpreted as being suggestive of MBL production.

Combined Disc Diffusion Test (CDDT):

- The strain to be tested was inoculated onto MHA plate as suggested by the CLSI. Two Imipenem(10µg) discs were placed on the plate and 10µl of 0.5 M EDTA solution was added to one of them to obtain the desired concentration (750µg).
- After18 hours of incubation, the zone diameter of Imipenem and Imipenem EDTA discs were compared. The increase in inhibition zone with Imipenem with EDTA disc ≥5mm than the Imipenem disc alone was considered as MBL positive.

- Similarly the test was done with two Ceftazidime discs and zone enhancement of ≥5mm with EDTA was considered as positive.

Double Disc Synergy Test (DDST):

- Lawn culture of the test organism was prepared over Mueller-Hinton agar plate as per CLSI guidelines.
- A plain sterile disc was kept 10 - 20 mm apart from either a Ceftazidime(30µg) or Imipenem(10µg) disc.
- 5 µl of EDTA was added to plain disc and incubation was done at 37˚C overnight.
- Presence of an extended zone from Imipenem or Ceftazidime disc towards EDTA was interpreted as positive.

Modified Hodge Test (MHT):

- ATCC E.coli 25922 inoculum was prepared in 0.5 Mc Farland standards & lawn culture was made on Mueller-Hinton agar plate.
- Meropenem disc (10µg) was kept in the centre of the lawn.
- Colonies of Meropenem or Ceftazidime resistant isolates were taken & inoculated from edge of the disc to edge of the plate in a straight line & incubation was done at 37° C over night.

- The formation of a clover leaf like indentation along the test strains indicated carbapenemase production.

MBL E test (Imipenem and Imipenem with EDTA):

- E test is a predefined, stable gradient of antibiotic concentrations on a plastic strip. Using innovative dry chemistry technology, E test is used to determine the on-scale Minimum Inhibitory Concentration (MIC) of antibiotics, antifungal agents and anti-mycobacterial agents.
- E test MBL strip is a unique phenotypic detection strip which is coated with mixture of Imipenem + EDTA and Imipenem in a concentration gradient manner. The strip is made of porous material and the antibiotics are distributed evenly on either side of the strip.
- E test MBL strip has a double sided antibiotic dilution range of Imipenem (4 to 256μg/ml) and Imipenem (1 to 64 μg/ml) in combination with a fixed concentration of EDTA.
- **Procedure**: The inoculum for E test was prepared as for other tests as mentioned previously. A sterile cotton swab was soaked into standardised inoculum and the swab rotated and pressed firmly against the inside wall of the tube to express excess fluid. The

entire agar surface of the Mueller-Hinton agar plate was streaked with the swab three times by turning the plate at 60˚ angle between each streaking and allowed to dry for 15 minutes. The E test MBL strip container was removed from the cold and kept at room temperature for 15 minutes before opening .The strip was then taken with a sterile forceps and applied to the dried agar surface with the MIC scale facing upwards. The strip adsorbed within 60 seconds to the agar surface and then incubation of plate was done at 37˚C for 24 hours and the results were interpreted.

- **Interpretation**: The plates were read only after sufficient growth was seen. The value of MIC was read where the ellipse intersected the scale on the strip.MIC ratio of Imipenem to Imipenem + EDTA of >8, or reduction of Imipenem MIC by ≥ 3 Log 2 dilutions in the presence of EDTA or formation of a phantom zone indicated MBL production.

DETECTION OF MBL PRODUCTION IN PSEUDOMONAS AERUGINOSA BY GENOTYPIC METHOD - PCR

IMP/ VIM Gene Assay

Requirements:

DNA purification kit, PCR Master Mix, Agarose gel electrophoresis and Primers.

2X PCR Master Mix:

It contains 2U of Taq DNA polymerase, 10X Taq reaction buffer, 2mM MgCl2, 1µl of 10mM dNTPs mix and PCR additives.

Agarose gel electrophoresis:

Agarose, 50X TAE buffer, 6X gel loading buffer and Ethidium bromide.

DNA extraction from *P.aeruginosa* isolates - Procedure:

Genomic DNA extraction from bacterial pellet using PureFast Genomic DNA purification kit:

1. Bacterial Pellet was suspended in 200µl of PBS.
2. 50µl of Lysozyme [50mg/ml] was added and incubation was done at 37ºC for 15min.
3. Added 400µl of Lysis Buffer and 40µl of Proteinase K [Reconstituted]
4. Mixed immediately by inverting and incubation was done at 70ºC for 10min.

5. Added 100µl of Isopropanol and mixed well.
6. Pipetted entire sample into the PureFast® spin column. Centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and the column was placed back into the same collection tube.
7. 500µl Wash Buffer-I was added to the PureFast® spin column. Centrifuged at 13,000 rpm for 30-60 seconds and discarded the flow-through. Placed the column back into the same collection tube.
8. Added 750µl Wash Buffer-II to the PureFast® spin column. Centrifuged at 13,000 rpm for 30-60 seconds and discarded the flow-through. Placed the column back into the same collection tube.
9. Repeated Step 8 once.
10. Discarded the flow-through and centrifuged at 13,000 rpm for an additional 1 min. This step was essential to avoid residual ethanol.
11. Transferred the PureFast® spin column into a fresh 1.5 ml micro centrifuge tube.
12. Added 100µl of the pre-warmed Elution Buffer to the PureFast® spin column.
13. Incubation was done for 2 min at room temperature and centrifuged at 13,000 rpm for 2 min.
14. Discarded the column and stored the purified DNA at -20°C. For gel analysis, loaded 10 - 20µl of elute.

PCR Procedure:

Primer Sequence:

IMP gene primer

Product size = 220bp

Forward Primer- 5'-TTTTGCAGCATTGCTACCGC-3'

Reverse primer- 5'-CACGCTCCACAAACCAAGTG-3'

VIM gene primer

Product size = 442bp

Forward Primer - 5'-GTGCTTTGACAACGTTCGCT-3'

Reverse primer - 5'-TCCACGCACTTTCATGACGA-3'

1. Reactions set up as follows;

VIM Setup:

Components	**Quantity**
In PCR vial Master mix	25µl
VIM Primer Mix (10pmoles/µl)	1µl
Genomic DNA	**1µl**
Water, nuclease free	23µl
Total volume	50µl

IMP Setup:

Components	**Quantity**
In PCR vial Master mix	25µl
IMP Primer Mix (10pmoles/µl)	1µl
Genomic DNA	**1µl**
Water, nuclease free	23µl
Total volume	50µl

2. Mixed gently and spinned down briefly.
3. Placed into PCR thermocycler and programmed as follows;

Initial Denaturation: 94ºC for 3 min

Denaturation: 94ºC for 1 min
Annealing: 58ºC for 1min
Extension: 72ºC for 1min
} 30 cycles

Final extension: 72º C for 5 min

Agarose gel electrophoresis:

1. Prepared 2% agarose. (2gm agarose in 100ml of 1X TAE buffer and melted using micro oven).
2. When the agarose gel temperature was around 60ºC, 5µl of Ethidium bromide was added.
3. Poured warm agarose solution slowly into the gel platform.
4. Kept the gel set undisturbed till the agarose solidified.
5. Poured 1XTAE buffer into submarine gel tank.
6. Carefully placed the gel platform into tank. Maintained the tank buffer level 0.5cm above the gel.
7. Templates were loaded after mixing with gel loading dye along with 10µl HELINI 100bp DNA Ladder.
8. Run electrophoresis at 50V till the dye reaches three fourth distance of the gel.

9. Gel viewed in UV Transilluminator and observed the bands pattern.

Interpretation: The presence of IMP and VIM gene was indicated by the amplification of 220 & 442 base pairs PCR product from the clinical isolates respectively.

The various phenotypic methods were compared with PCR and sensitivity, specificity, PPV and NPV were determined. The P value and 95% confidence intervals were obtained using SPSS (Stastical Package for Social Sciences) version 16. P value <0.05 was taken as significant and P value >0.05 indicates that there is no significant difference between the various tests compared and PCR.

RESULTS

A total of 580 clinical samples of pus, sputum, urine, blood, ascitic fluid, endotracheal fluid, broncho-alveolar lavage and wound swab collected from the patients admitted to various clinical departments of Govt.Rajaji Hospital Madurai were processed and the results are shown in Table no.1.

Table 1. Specimen wise isolation of organisms n=580

Specimen	P.aeruginosa	Other Gram negative bacilli	Gram positive cocci	No growth	Total
Pus	33(5.68%)	32(5.51%)	90(15.51%)	20(3.44%)	175(30.17%)
Wound swab	24(4.13%)	48(8.27%)	30(5.17%)	12(2.06%)	114(19.65%)
Sputum	20(3.44%)	22(3.79%)	18(3.10%)	-	60(10.34%)
Urine	18(3.10%)	70(12.06%)	10(1.72%)	23(3.96%)	121(20.86%)
Blood	7(1.20%)	17(2.93%)	52(8.96%)	12(2.06%)	88(15.17%)
Body fluids	6(1.03%)	13(2.29%)	-	3(0.51%)	22(3.79%)
Total	**108**(18.62%)	**202**(34.82%)	200(34.08%)	70(12.06%)	**580**

Out of the 580 clinical samples, 175 samples were pus, 114 samples were wound swab, 121 samples were urine, 60 samples were sputum,88 samples were blood and 22 samples were body fluids.70

samples showed no growth and 510 showed growth. Among the 510 isolates, 108 were *Pseudomonas aeruginosa*, 202were Gram negative bacilli other than Pseudomonas, 200 were Gram positive cocci. The analysis of specimen wise, age wise and sex wise distribution of Pseudomonas aeruginosa was done and given in Table no. 2, 3 & 4.

Table2. Specimen wise isolation of Pseudomonas aeruginosa

n =108

Specimen	No. of Pseudomonas aeruginosa isolates	Percentage (%)
Pus	**33**	**30.5**
Wound Swab	**24**	**22.2**
Sputum	20	18.5
Urine	18	16.6
Blood	7	6.4
Body fluids	6	5.5

Among the 108 *Pseudomonas aeruginosa* isolates, 33 were from pus, 24 from wound swab, 20 from sputum, 18 from urine, 7 from blood and 6 from body fluids.

Table3. Age wise distribution of P.aeruginosa n =108

Age in years	Number of patients	Percentage (%)
< 1	1	0.92
1-10	12	11.1
11-20	9	8.33
21-30	19	17.59
31-40	**20**	**18.51**
41-50	**21**	**19.44**
51-60	13	12.03
>60	13	12.03

Age wise distribution of Pseudomonas aeruginosa was analysed which showed 1 isolate was from less than 1 year of age, 12 were from 1-10 years age group, 9 from 11-20 years age group, 19 from 21-30 years age group, 20 from 31-40 years age group, 21from 41-50 years age group13 from 51-60 years age group, and 13 from >60 years age group.

Table4. Sex distribution of P.aeruginosa n=108

Male	Female`
61(56%)	47(44%)

Out of the 108 isolates of P.aeruginosa, 61were isolated from males and 47 were isolated from females.

Table5. Risk factors associated with P.aeruginosa n = 108

Risk factors	No. of P.aeruginosa isolates	Percentage (%)
Burns	**24**	**22.2**
Ventilator associated pneumonia	**12**	**11.1**
Diabetes	8	7.4
Otitis externa	4	3.7
Keratitis	1	0.9
Malignancy	1	0.9

On analyzing various risk factors associated with Pseudomonas aeruginosa, 24 isolates were associated with burns, 12 were associated with ventilator associated pneumonia, 8 were associated with diabetes, 4

were associated with otitis externa, 1 was associated with keratitis and 1 with malignancy.

Table6. Antimicrobial susceptibility testing showing resistance pattern of P.aeruginosa by Disc diffusion method n = 108

Antibiotic discs	Zone size (mm)			No .of resistant Isolates	Percentage (%)
	Resistance (mm/less)	Intermediate (mm)	Sensitive (mm/more)		
Gentamicin (GEN) 10 µg	12	13-14	15	42	39
Amikacin (AK)30 µg	14	15-16	17	80	74
Norfloxacin (NX) 10 µg	12	13-16	17	65	60
Ciprofloxacin (CP) 5 µg	15	16-20	21	77	71
Netilmicin (NET) 30 µg	12	13-14	15	44	41
Ceftazidime (CAZ)30 µg	14	15-17	18	24	**22**
Ceftriaxone (CTR) 30 µg	13	14-20	21	24	22
Imipenem (IPM) 10 µg	13	14-15	16	20	**18.5**
Meropenem (MRP) 10 µg	13	14-15	16	20	**18.5**
Piperacillin+ Tazabactam (PT) 100/10 µg	17	-	18	68	63
Piperacillin (PI) 100 µg	17	-	18	81	75
Polymyxin B (PB) 300 µg	11	-	12	0	0

Antimicrobial susceptibility testing of *Pseudomonas aeruginosa* isolates to various drugs like Piperacillin, Piperacillin-Tazabactam, Gentamycin, Amikacin, Norfloxacin, Ciprofloxacin, Netilmicin Ceftazidime, Ceftriaxone, Imipenem, Meropenem, and Polymyxin B was done. The percentage of *P.aeruginosa* isolates resistant to various drugs are shown in the above table.

Table 7. MIC determination of P.aeruginosa by agar dilution method.

n = 108

Imipenem (µg/ml)		Ceftazidime (µg/ml)	
<8	>8	<32	>32
90(83.44%)	**18 (16.66%)**	88 (51.19%)	**20 (18.5%)**

Out of the 108 isolates, MIC determination by agar dilution method showed 18 isolates were having >8 MIC for Imipenem and 20 isolates were having >32 MIC for Ceftazidime.

Table 8. Number of isolates showing fourfold reduction in MIC with EDTA by agar dilution method n=20

Imipenem with EDTA	Ceftazidime with EDTA
18 (90%)	19 (95 %)

By agar dilution method the MIC of Imipenem and Ceftazidime along with EDTA was determined. The Table above shows the number of isolates which shows fourfold reduction in MIC with EDTA when combined with Imipenem and ceftazidime.

18 isolates showed fourfold reduction with Imipenem when combined with EDTA and 19 isolates showed reduction of four fold in MIC with Ceftazidime when combined with EDTA.

Table 9. Metallo-β-lactamases detection by various phenotypic methods

n=20

Method	Agar dilution(MIC)		E test (MIC)	Modified Hodge test	Double Disc SynergyTest		Combined Disc Diffusion Test	
	I+EDTA	CAZ+EDTA			I+EDTA	CAZ+EDTA	I+EDTA	CAZ+EDTA
No. Of isolates & %	18(90%)	**19(95%)**	**19(95%)**	12(60%)	17(85%)	18(90%)	17(85%)	**19(95%)**

Out of the 20 isolates which were screening positive, Agar dilution (IMP + EDTA) detected 18 isolates, Agar dilution (CAZ+ EDTA) detected 19 isolates, Modified Hodge test detected 12 isolates, E test detected 19 isolates, DDST(I+EDTA) detected 17 isolates, DDST (CAZ+EDTA) detected 18 isolates, CDDT (I+EDTA) detected 17 isolates and CDDT (CAZ+EDTA) detected 19 isolates as MBL producers.

Table10. MBL detection by Genotypic method (PCR) n= 20

Gene	IMP alone	IMP & VIM (Both)	Total positives
No. of isolates amplified	**17(85%)**	**1(5%)**	**18 (90%)**

Out of the 20 isolates subjected to PCR, 17 isolates were positive for IMP gene and 1 isolate was positive for both IMP and VIM gene.

Table No.11 Age distribution of MBL producing Pseudomonas aeruginosa isolates n = 18

Age	No. of isolates	Percentage (%)
<1	1	5.55
1-10	1	5.55
11-20	2	11.11
21-30	2	11.11
31-40	**8**	**44.44**
41-50	1	5.55
51-60	2	11.11
>60	1	5.55

Out of the 18 isolates, 1 isolate was form <1 year age group, 1 from 1-10 years, 2 from 11-20 years, 2 from 21-30 years,8 from 31-40 years,1 from 41-50 years, 2 from 51-60 years and 1from > 60 years age group.

Table .12 Sex distribution of MBL producing Pseudomonas aeruginosa isolates n = 18

Male	Female
13(72.22%)	5(27.78%)

The sex distribution of MBL producing *Pseudomonas aeruginosa* in the clinical isolates was analysed and 13 isolates were from male patients and 7 isolates were from female patients.

Table .13 Specimen wise distribution of MBL producing Pseudomonas aeruginosa n=18

Specimen	No. of isolates	Percentage (%)
Pus	3	16.67
Wound swab	**9**	**50.00**
Sputum	3	16.67
Urine	2	11.11
Body fluids	1	5.56

The specimen wise distribution of MBL producing Pseudomonas was analysed and MBL positive isolates in pus sample were 3, 9 from wound swab, 3 from sputum, 2 from urine and 1 from body fluids.

Table14. Risk factors associated with MBL production n=18

Risk factors	No. of isolates	Percentage (%)
Burns	**8**	**44.44**
On prolonged Ventilatory support	**4**	**22.22**
Diabetes	2	11.11
Malignancy	1	5.56
Surgery/Post op wound infection	1	5.56
Longer Hospital stay	1	5.56
Prolonged use of Antibiotics	1	5.56

On analysis of the various risk factors associated with MBL production in pseudomonas, it was observed that 8 isolates were associated with burns, 4 with prolonged ventilatory support 2 with diabetes, 1 with malignancy, 1 with surgery/post op wound infection, 1 with longer hospital stay and 1 with prolonged use of antibiotics.

Various phenotypic methods were compared with PCR and the Sensitivity, Specificity, PPV and NPV are shown in Tables 15 to22.

Table15. Comparison of **Modified Hodge Test** with PCR

		PCR			Sensitivity(%) With 95%CI	Specificity(%) With 95% CI	PPV(%) With 95%CI	NPV(%) With 95%CI
		positive	negative	Total				
MHT	Positive	12(100%)	0(0%)	12(100%)	67% (44-84)	100%(34-100)	100% (76-100)	25% (7-59)
	negative	6(75%)	2(25%)	8(100%)				
	total	18(90%)	2(10%)	20(100%)				

P value: 0.147.

Table16. Comparison of **Double Disc Synergy Test (IPM+EDTA)**

with PCR

		PCR			Sensitivity With 95%CI	Specificity With 95% CI	PPV With 95%CI	NPV With 95%CI
		positive	negative	Total				
DDST (IPM+ EDTA)	Positive	16(94.1%)	1(5.9%)	17(100%)	89% (67-97)	50%(9-91)	94% (73-99)	33% (6-79)
	negative	2(66.7%)	1(33.3%)	3(100%)				
	total	18(90%)	2(10%)	20(100%)				

P value: 0.284.

Table17. Comparison of **Double Disc Synergy Test (CAZ+EDTA)**

with PCR

		PCR			Sensitivity With 95%CI	Specificity With 95% CI	PPV With 95%CI	NPV With 95%CI
		positive	negative	Total				
DDST (CAZ+ EDTA)	Positive	17(94.4%)	1(5.6%)	18(100%)	94% (74-99)	50%(9-91)	94% (74-99)	50% (9-91)
	negative	1(50%)	1(50%)	2(100%)				
	total	18(90%)	2(10%)	20(100%)				

P value: 0.195.

Table18. Comparison of **Combined Disc Diffusion Test (IPM+EDTA)**

with PCR

		PCR			Sensitivity With 95%CI	Specificity With 95% CI	PPV With 95%CI	NPV With 95%CI
		positive	negative	Total				
CDDT (IPM+ EDTA)	Positive	16(94.1%)	1(5.9%)	17(100%)	94% (74-99)	50%(9-91)	94% (74-99)	50% (9-91)
	negative	2(66.7%)	1(33.3%)	3(100%)				
	total	18(90%)	2(10%)	20(100%)				

P value: 0.284.

Table19. Comparison of **Combined Disc Diffusion Test (CAZ+EDTA)**

with PCR

		PCR			Sensitivity With 95%CI	Specificity With 95% CI	PPV With 95%CI	NPV With 95%CI
		positive	negative	Total				
CDDT (CAZ+ EDTA)	Positive	18(94.7%)	1(5.3%)	19(100%)	100% (74-99)	50%(9-91)	95% (75-99)	100% (21-100)
	negative	(0%)	1(100%)	3(100%)				
	total	18(90%)	2(10%)	20(100%)				

P value : 0.100.

Table20. Comparison of **E Test (IPM+EDTA)** with PCR

		PCR			Sensitivity With 95%CI	Specificity With 95% CI	PPV With 95%CI	NPV With 95%CI
		positive	negative	Total				
E test	Positive	18(94.7%)	1(5.3%)	19(100%)	100% (82-100)	50%(9-91)	95% (75-99)	100% (21-100)
	negative	0(0%)	1(100%)	1(100%)				
	total	18(90%)	2(10%)	20(100%)				

P value : 0.100.

Table21. Comparison of **Agar Dilution method (IPM+EDTA)** with PCR

		PCR			Sensitivity With 95%CI	Specificity With 95% CI	PPV With 95%CI	NPV With 95%CI
		positive	negative	Total				
Agar Dilution method (IPM+ EDTA)	Positive	17(94.4%)	1(5.6%)	18(100%)	94% (74-99)	50%(9-91)	94% (74-99)	50% (9-91)
	negative	1(50%)	1(50%)	2(100%)				
	total	18(90%)	2(10%)	20(100%)				

P value : 0.195.

Table22. Comparison of **Agar Dilution method (CAZ+EDTA)** with PCR

		PCR			Sensitivity With 95%CI	Specificity With 95% CI	PPV With 95%CI	NPV With 95%CI
		positive	negative	Total				
Agar Dilution method (CAZ+ EDTA)	Positive	18(94.7%)	1(5.3%)	19(100%)	100% (82-100)	50%(9-91)	95% (75-99)	100% (21-100)
	negative	0(0%)	1(100%)	1(100%)				
	total	18(90%)	2(10%)	20(100%)				

P value :0.100.

Table23. Comparison of **PCR** with other **Phenotypic methods** n=20

Methods	True positives	False negatives	False positives	True negatives
PCR	18	-	-	2
Agar dilution(IPM+EDTA)	17	1	1	1
Agar dilution(CAZ+EDTA)	18	0	1	1
E test	18	0	1	1
MHT	12	6	0	2
DDST(IPM+EDTA)	16	2	1	1
DDST(CAZ+EDTA)	17	1	1	1
CDDT(IPM+EDTA)	16	2	1	1
CDDT(CAZ+EDTA)	18	0	1	1

Table24. Sensitivity and Specificity of various Phenotypic methods

Methods	Sensitivity	Specificity	PPV	NPV	P value
Agar dilution(IPM+EDTA)	94	50	94	50	0.195
Agar dilution(CAZ+EDTA)	100	50	95	100	0.100
E test	100	50	95	100	0.100
MHT	67	100	100	25	0.147
DDST(IPM+EDTA)	89	50	94	33	0.284
DDST(CAZ+EDTA)	94	50	94	50	0.195
CDDT(IPM+EDTA)	89	50	94	33	0.284
CDDT(CAZ+EDTA)	100	50	95	100	0.100

DISCUSSION

Pseudomonas aeruginosa is one of the leading causes of nosocomial infections giving rise to a wide range of life threatening conditions. The intrinsic resistance exhibited by *Pseudomonas* to a wide range of antibiotics imposes a serious therapeutic problem. Carbapenems are useful antimicrobial agents for the treatment of infections caused by *Pseudomonas aeruginosa.* However, with the increasing use of these compounds to treat the life threatening conditions, Carbapenem resistant *P.aeruginosa* has developed. The prevalence of metallo-beta-lactamase, one of the carbapenemases in *Pseudomonas* is of serious concern in the recent years worldwide. As there are currently no standard guidelines described so far, several studies have been undertaken worldwide to detect the MBL production. Hence, early identification of MBL production is of great clinical significance in combating the resistance. In view of this, the present study was undertaken to identify a simple and reproducible screening method to detect MBL in this institution.

A total of 580 samples were processed in the present study, the predominant isolate among all the isolates were the Gram-negative bacilli and out of 580 samples, 202(34.82%) were Gram-negative bacilli and 108(18.62%) were *P.aeruginosa.* Out of the 108 *P.aeruginosa* , more

number of isolates 33(30.5%) were isolated from pus samples followed by 24(22.2%) from wound swab. It was also inferred that more number of Pseudomonas isolates were isolated from 41- 50 years (19.44%) age group followed by 31-40 years (18.51%) age group. Analysis of sex wise distribution of Pseudomonas infection showed that most of Pseudomonas infections were associated with males (56%) than females (44%). A recent study done by Ramprasad Balikaran et al.,(2010) [111] also reported the highest incidence of *Pseudomonas aeruginosa* were among males than females.The various risk factors associated with Pseudomonas infection were analysed and it was inferred that *P.aeruginosa* were predominantly isolated 24(22.2%) from burns patients followed by 12(11.1%) from patients on ventilatory support . Seema Bose et al., 2012[120] studied the incidence of MBL in *P.aeruginosa* in burns patients for a period of nine months and explained the high rate(15.71%) of infection in burns patients with Pseudomonas could be due to the loss of protective barrier of skin and the presence of devitalised tissues enhancing the growth of microorganisms and inhibiting the penetration of systemically administered antibiotics mediating drug resistance.

In India, the prevalence of Metallo-Beta-Lactamases in Pseudomonas ranging from 8 to 14% has been documented (Hirakata et al., 1998; Varaiya et al., 2008; Zavascki et al., 2006) [47,130,143].

Various studies have reported the use of different methods for the detection of MBLs like Imipenem-EDTA combined disc test, Double disc synergy test using Imipenem-EDTA and Ceftazidime with EDTA, E test and Modified Hodge test (Behera et al.,2008;Arakawa et al.,2000;Lee et al.,2003;Yong et al.,2002;) [9,7,66,140].

In this study, MBL screening and confirmation was done by phenotypic and genotypic methods. The phenotypic methods used were the MIC determination by Agar dilution method using Imipenem and Ceftazidime in combination with EDTA to detect the four fold reduction in MIC, Combined disc diffusion test using Imipenem and Ceftazidime with EDTA, Double disc synergy test using Imipenem and Ceftazidime with EDTA, E test to detect MIC and Modified Hodge test. These phenotypic methods were compared with the genotypic method for sensitivity and specificity.

With the Phenotypic and genotypic methods used to detect MBL in this study, it was found that **18.5%(18/108)** isolates were resistant to Imipenem, **20.37%(20/108)** were resistant to Ceftazidime and the prevalence of MBL in this institution was found to be **16.66%** which is slightly closer to prevalence reported by Hemalatha et al., 2005[44] and Seema et al., 2012[120].

Bashir et al., 2011[12] reported that out of the 132(46.6%) P.aeruginosa isolates, 38(13.42%) were resistant to Imipenem and out of which 33(**11.66%**) were MBL producers. Hemalatha et al., 2005[44] reported 16% of *Pseudomonas aeruginosa* isolates were resistant to Imipenem and **14 %** were positive for MBL production by Combined disc test. Behera et al., (2008)[9] reported 14.47% of *P.aeruginosa* were resistant to Imipenem and **10.53%** positive for MBL production by combined disc test. Agrawa et al., [1] reported 8.05% Imipenem resistance and 10.35% Ceftazidime resistance in MBL producers. Sarkar et al., 2006 [117] documented 36.36% resistance to Imipenem and **53.2%** were MBL positive. Navaneeth et al., 2002[90] reported 12% MBL mediated Imipenem resistance in P.aeruginosa. Mihani et al., 2007[86] documented **19.51%** Imipenem resistant P.aeruginosa strains isolated from burns patients were MBL producers. Seema et al., 2012[120] reported 30% Imipenem resistance, out of which 20(14.28%) were non-MBL producers and **22(15.71%)** were MBL producers. But in this study **44.44 %** of burned patients infected with *P.aeruginosa* were MBL producers which were slightly higher than the other studies.

Bashir et al., 2011[12] reported the mean age of the patients from whom MBL producers were isolated was 50.66 years. The highest number of cases was above 60 years of age. In this study, most of the

MBL producing isolates were from the age group 31- 40 years(44.44%) showing a lower preponderance in contrast to the age documented by Bashir et al.,2011 [12]. This could be due to the fact that most of the male patients inflicted with burns were in the 31 – 40 years age group and the suicidal risk is more common in that age group.

Out of 18 patients from whom MBL producers were isolated in this study, 13(72.22%) were males and 5(27.78%) were females and similar male preponderance was documented by Bashir et al., 2011 [12] also and in their study 51.5% were males and 48.5% were females which was not statistically significant (P>0.05). Debasrita Chakraborty et al., 2010[26] also documented that male patients were predominantly affected with MBLs than females.

In this study, the predominant source of MBL positive strains was from wound swab 9(50%), followed by sputum 3(16.67%), pus 3(16.67%), urine 2(11.11%) and body fluids 1(5.56%). Hirakata et al., 1998[47] reported that the predominant source of MBL as urinary tract (40%). Bashir et al., 2011[12], documented the predominant source of MBL positive strain from urinary tract 9(27.3%) followed by wound swab 2(24.2%) blood 6(18.2%), sputum 4(12.1%),CSF 3(9.1%),pus 2(6.1%) and endotracheal tip 1(3%).

This shows that the predominant source of MBL positive strain was wound swab in our study which is in total contrast to study conducted by Bashir et al[12] and Hirakata et al[467] where the urinary tract was the predominant source.

On analysis of the various risk factors associated with MBL production in pseudomonas in this study, it was inferred that 44.44% isolates were from burns patients, 22.22% from patients with prolonged ventilatory support, 11.11% from diabetic patients, 5.56 % from malignancy, 5.56 % surgery/post op wound infection, 5.56 % from prolonged hospital stay and 5.56 % with prolonged use of antibiotics. Bashir et al.,2011[12] reported that 24.2% of MBLs were isolated from patients with malignancy,15.2% from patients with diabetes,15.2% from patients with sepsis,12.1% from patients with cardiovascular disease,12.1% from patients with CNS infections,12.1% from patients with trauma,9.1% from patients with burns. This indicates that burns patients have significant risk association for MBL in the current study which was not significantly associated with study conducted by Bashir et al [12] who documented burns as the least risk factor for MBL production. But Seema Bose et al., 2012[120] reported the incidence of 15.71% of MBL from burns patients. So, it was inferred that burns wound has risk

association with MBL and the prevalence was found to vary in different health care settings.

In this study the antimicrobial susceptibility of various *P.aeruginosa* isolates revealed varied levels of resistance to antipseudomonal penicillins, third generation cephalosporins, aminoglycosides and fluoroquinolones. Polymyxin B was the most effective antibiotic in treating P.aeruginosa infections with no documented resistance which was acknowledged in study conducted by Sarkar et al.,2006 [117].

In the present study, the resistance to Imipenem and Meropenem were 18.5% for both the drugs and 22% resistance was recorded for Ceftazidime by Kirby-Bauer disc diffusion method and 18.5% for Ceftazidime and 16.66% for Imipenem by MIC agar dilution method which was in close concordance with Shahid et al.,2004 [122] who reported 20% Ceftazidime resistance. In the present study, 4 isolates which showed resistance for Ceftazidime by disc method were sensitive by Agar dilution method and 2 isolates which showed resistance for Imipenem by disc diffusion method were sensitive by Agar dilution method. MIC was also determined by combining EDTA with Imipenem and Ceftazidime individually to note the four fold reduction in MIC which indicated MBL production. It was inferred that 100% of the

isolates which showed resistance with disc method showed 4 fold reduction in MIC with EDTA for Imipenem but for Ceftazidime, 1 isolate out of the 20 which showed resistance did not show 4 fold reduction in MIC with EDTA indicated that some other resistance mechanism (could be ESBL) for Ceftazidime resistance other than MBL production.

Pitt et al.,2003[109] reported 39.6% Ceftazidime resistance in their study and Debasrita Chakraborty et al.,2010[26] reported 100% resistance to third generation cephalosporins (Ceftazidime, Cefotaxime and Ceftriaxone) in MBL positive isolates of *P.aeruginosa* and 78.25% resistance for Imipenem in a study conducted in ICU patients. Agrawal et al., 2008[1] reported 40.22% resistance to Piperacillin. Sarkar et al., 2006 [117] and Pitt et al., 2003[109] observed 12% and 31.9% resistance against Piperacillin respectively. In the present study, 75% resistance was observed against Piperacillin which is much higher from the other studies. This may be due to the indiscriminate use of this drug in our locality which has resulted in higher percentage of resistance.

Agrawa et al.,2008 [1] reported least resistance with Amikacin whereas Sarkar et al [117] found 40.90% resistance with Amikacin,45.45% with Gentamicin,59.09% with Tobramycin, and 50.5% with Netilmycin.

In this study, 74% resistance with Amikacin, 60% with Norfloxacin, 71% with Ciprofloxacin, 22% with Ceftriaxone, 39% with Gentamicin,

41% with Netilmycin, and 0% with Polymyxin B has been detected. It is inferred that the Changing resistance patterns in clinical isolates of *P.aeruginosa* in various localities as indicated by various studies indicate the fact that the commonly used antipseudomonal agents like Aminoglycosides, Quinolones and few third generation Cephalosporins show a higher percentage of resistance than the newer third generation cephalosporins like Ceftazidime and Carbapenems (Imipenem and Meropenem).Hence, the frequent and inadvertent usage of the different groups of drugs have resulted in increasing resistance patterns in the isolates and there seems to be emergence of resistance in the newer group of drugs like few Cephalosporins and Carbapenems also. But Polymyxin B, which was the older drug has not been used frequently due to the drug toxicity showed 0% resistance pattern and found to be the one of the fewer options available in pipeline to treat the emerging MBL positive isolates of P.aeruginosa.

Nirav V.Pandya et al (2011) [96] used various phenotypic methods on isolates which were screened for MBL by Kirby-Bauer disc diffusion method. They isolated 27 out of 450 isolates as screening positive and further proceeded with phenotypic methods and reported 96.3% were MBL positive by combined disc diffusion test using Imipenem and EDTA and 81.48% were MBL positive by double disc synergy test using

Imipenem and EDTA. 85.19% positive by combined disc diffusion test using Ceftazidime and EDTA, 44.44% positive by double disc synergy test using Ceftazidime. They reported a prevalence of 9.92% MBL. They inferred that CDDT using Imipenem followed by CDDT using Ceftazidime as the most sensitive methods in the detection of MBLs .On comparing the various published studies with the present study, the following interpretation was made.

STUDY	CDDT-Imipenem	DDST-Imipenem	CDDT-Ceftazidime	DDST-Ceftazidime
Picao et al[106]	80%	**82.6%**	**83%**	45.7%
Galani et al[34]	94.7%	**100%**	**100%**	77.9%
Franklin et al[32]	**100%**	79%	-	-
Present study	89%	89%	**100%**	**94%**

In the present study, **CDDT- CAZ and DDST-CAZ** could detect additional isolates than CDDT-IMP AND DDST-IMP. This indicates that Ceftazidime is a better screening drug for MBL detection than Imipenem. With the emergence of **Carbapenem sensitive MBLs** as described by Yan et al 2004 [138] and Clare Franklin et al 2006 [20] . **MBL genes which are hidden** mediate MBL production where the **isolates**

show resistance to Ceftazidime but are susceptible to Carbapenems. Similarly, Hemalatha et al., (2005) [44] found that Ceftazidime with EDTA could pick up additional isolates of MBL producers. But Bashir et al 2011[12] picked up more MBL positive isolates by Imipenem with EDTA than with Ceftazidime EDTA discs. This could be explained by the fact that MBL producing organism might have some other Ceftazidime resistance mechanisms. So, with such isolates ,CDDT using Ceftazidime will not show MBL production as suggested by Behera et al[9].In our study, Ceftazidime seems to be a better drug in the detection of MBLs than Imipenem and yet another fact that Imipenem is an unstable drug that might miss MBLs, owing to their susceptibility when tested alone.

Bashir et al., 2011[12] reported 100% positive results with E Test. Varaiya et al., 2008 [130] and Pitout et al., 2005 [108] reported 96% sensitivity with E test. In the present study, E test showed 100% sensitivity similar to study conducted by Bashir et al.,2011[12].

Lee et al.,2003[66] used Hodge test as a simple screening test for MBL producing isolates ,but occasional isolates showed false negative results. Rizvi et al., 2009[116] found DDST using EDTA better than Hodge test. Attal Ro et al., 2010[8] reported two Imipenem resistant strains were found to be Carbapenem hydrolysis negative by the Hodge test and

Modified Hodge test and the likely reason for the Imipenem resistance may be a mechanism other than Carbapenem hydrolysis such as decreased membrane permeability. They also used 50 mM of zinc sulphate to improve sensitivity of Hodge test & added 10 μl of 50 mM zinc sulphate to Imipenem discs for Modified Hodge test, DDST and CDDT. Similarly in this study, only 12 out of the 20 isolates were positive by Modified Hodge test showing the least sensitivity to identify MBLs when compared to other methods.

Seema bose et al., 2012[120] showed that DDST and MBL E test were both equally sensitive and easy to perform. Behera et a.,2008l[9] used four different methods for MBL detection and found that CDDT using Imipenem and EDTA as superior to DDST using Imipenem and EDTA and Cetazidime with EDTA as DDST had the major disadvantages of subjective interpretation of results in some instances. This is in concordance with the other published studies which have found that CDDT to be one of the most sensitive techniques for detecting MBLs. They also suggested with the emergence of Carbapenem sensitive MBLs, the concern of which isolates to be selected for phenotypic MBL detection is controversial and hence they suggested that the combined

disc method to be applied on all isolates which show resistance to Ceftazidime and Ticarcillin with Clavulanic acid.

Agrawal et al.,2008[1] and Arakawa et al.,2000 [7] inferred that MBL producer may be Imipenem sensitive but might have high minimum inhibitory concentration for Ceftazidime and concluded that Ceftazidime could be better choice for detection of MBL production. Arakawa et al.,2000 [7] recommended the testing of the Ceftazidime resistant isolates for MBL production, since some MBL producing Gram negative bacteria were inhibited by the low concentration of Imipenem and they were difficult to detect and also desctribed that IMP -1 producers usually exhibit high level resistance to Ceftazidime and have a significant inhibitory effect on thiol compounds. But Lee et al., 2003[66] reported that in their study, not a single MBL producing isolate was detected among the Imipenem sensitive isolates. In Japan, Sugino et al used only Carbapenem resistant isolates for the screening of MBLs.

Though various phenotypic methods have been described so far in various studies for the detection of MBLs, an accurate method to detect the MBLs with high sensitivity and specificity has not been proved so far. PCR analysis was described as a reliable and accurate method in various studies by Franklin et al.,2006 [32] and Arakawa et al..,2000[7] and Pitout

et al.,2005[108] performed a duplex PCR assay with high sensitivity and specificity that could simultaneously detect both VIM and IMP genes and also described that PCR confirmation for MBLs to be performed as an important method in regional laboratories, as EDTA can give false positive results in IMP resistant isolates due to altered OprD levels as described by Conejo et al., [23] . Pandya et al., 2011 [96] and Conejo et al., [23] also described PCR as the gold standard test for the detection of MBL production.

Hence, considering PCR as the gold standard, IMP and VIM gene assay was done and out of the 20 isolates subjected, 17 isolates were positive for IMP gene alone and one isolate was positive for both IMP and VIM gene. From the genotypic detection method, 18(16.66%) isolates were confirmed to be MBL producers. 2 isolates which were not detected in this study might be carrying some other genes other than IMP and VIM genes. Senda et al.,1996[121] also documented that IMP and VIM as the most predominantly prevalent genes in MBL positive isolates.In this study, the various phenotypic methods were compared with PCR and Sensitivity, Specificity, PPV and NPV were determined.

On comparison of various phenotypic tests with PCR, it was inferred that Agar dilution method (IPM+EDTA) had 94% sensitivity, 50%

specificity, 94%PPV, 50% NPV and P value 0.195. Agar dilution method (CAZ+EDTA) had 100% sensitivity, 50% specificity, 95%PPV, 100% NPV and P value 0.100. E test had 100% sensitivity, 50% specificity, 95%PPV, 100% NPV and P value 0.100. Modified Hodge Test test had 67% sensitivity, 100% specificity, 100%PPV, 25% NPV and P value 0.147. DDST (IPM+EDTA) had 89% sensitivity, 50% specificity, 94%PPV, 33% NPV and P value 0.284. DDST (CAZ+EDTA) had 94% sensitivity, 50% specificity, 94%PPV, 50% NPV and P value 0.195. CDDT (IPM+EDTA) had 89% sensitivity, 50% specificity, 94%PPV, 33% NPV and P value 0.284. CDDT (CAZ+EDTA) had 100% sensitivity, 50% specificity, 95%PPV, 100% NPV and P value 0.100. **Since P value in all the tests were not <0.05, it was inferred that there was no statistically significant difference between PCR and other phenotypic methods in the detection of MBLs.** Out of the 20 isolates which were resistant to Imipenem and Ceftazidime, PCR detected 18 isolates as positive. Agar dilution method using Ceftazidime+ EDTA, E test and CDDT (CAZ+EDTA) also detected the same 18 isolates as positive showing their high sensitivity in detecting all the MBL positive isolates. Modified Hodge Test detected only 12 isolates as positive, indicating its least sensitivity when compared to PCR but there was no false positives and detected all the true negatives indicating high

specificity. All the phenotypic methods detected one isolate as falsely positive except MHT. Conejo et al., [23] explained that false positives have been reported with EDTA-based methods due to the effect of EDTA on a zinc-dependent OprD pump which is responsible for Carbapenem entry in the isolates. Hence in this study, three phenotypic methods i.e., CDDT(CAZ+EDTA), E test, Agar dilution method using Ceftazidime + EDTA have equal sensitivity 100%, followed by DDST(CAZ+EDTA) & Agar dilution method (IPM+EDTA) 94% and DDST(IPM+EDTA) & CDDT(IPM+EDTA) 89% sensitivity. On analyzing the pros and cons of various methods, it is inferred that E test, though a highly sensitive method could not be done in all laboratories due to cost constraints and Agar dilution method being a cumbersome procedure and time consuming could not be routinely employed for MBL detection in all laboratories.

CDDT (CAZ+EDTA) is a simple, cost effective and easiest method with sensitivity very close to PCR in detecting all the MBL positive isolates. It was also inferred that an MBL producer may be Imipenem sensitive but might have high MIC for Ceftazidime and hence Ceftazidime is a better drug for MBL detection as described by Arakawa et al., 2000[7]. As PCR cannot be done in every laboratory due to its high

cost and the need for a sophisticated set up and trained personnel, the introduction of a simple and cost effective method to detect all the MBLs is the need of the hour in any diagnostic Microbiology laboratory for effective infection control and to prevent therapeutic failure and also to prevent dissemination of resistant strains in the health care settings as well as in the community.

SUMMARY

- The study showed a preponderance of Gram negative infections (53.44%) among various samples and out of which 18.63% were Pseudomonas aeruginosa.
- Among the Pseudomonas aeruginosa isolates 16.66% were MBL producers.
- 50% of MBL producers were isolated from wound swab collected from burns wounds.
- 44.44% of MBL producers were from 31-40 years and 5.55%of MBL producers were equally distributed in the extremes of age group.
- 72.22% of males and 27.78% of females were MBL producers showing male predominance.
- MBL has been most commonly associated with risk factors like burns (44.44%) and ventilator associated pneumonia (22.22%).
- Various phenotypic methods were used for the detection of MBLs and compared with PCR (IMP & VIM gene assay) as gold standard.

- Out of the 20 screening positive isolates, IMP gene alone was detected in 85% of isolates and both IMP & VIM genes were detected in 5% of isolates.
- CDDT (CAZ+EDTA), E test, Agar dilution method using Ceftazidime + EDTA have equal sensitivity of 100% in detecting MBLs and are as sensitive as PCR.
- CDDT (CAZ+EDTA) is a simple, cost effective and easiest method with sensitivity very close to PCR in detecting all the MBL producing *P.aeruginosa* isolates.
- Carbapenem sensitive but Ceftazidime resistant MBL positive isolates were detected and Ceftazidime was found to be the better substrate drug in detecting all the MBLs in the present study.

CONCLUSION

- The emergence of Gram negative bacterial species with acquired resistance to various broad spectrum Beta lactam antibiotics has become a clinical problem worldwide and particularly Pseudomonas is notorious for causing a wide range of hospital acquired infections.
- In this study, **Carbapenem sensitive MetalloBetaLactamases** in Pseudomonas aeruginosa have been found emerging predominantly from **burns ward** in Govt. Rajaji hospital, Madurai with a prevalence of **16.66%** with a predilection to affect males of the middle age group (31- 40 years).
- Early detection of these strains is crucial to establish an appropriate antimicrobial therapy and to prevent interhospital and intrahospital dissemination and thereby reduce the mortality and morbidity associated with these infections.
- On comparison of various phenotypic methods, **Combined Disc Diffusion Test using Ceftazidime and EDTA** was found to be the simple, cost effective and highly sensitive method close to PCR to detect all the carbapenem susceptible MBLs with hidden MBL genes which are a serious threat to infection control efforts.

- Formulating antibiotic policy, judicial use of higher antibiotics, isolation of MBL infected patients, strict safety precautions such as use of gloves, masks gowns and proper hand washing techniques are the measures that can be taken to combat the serious therapeutic challenge faced with MBL producing isolates.
- With only a fewer drugs available in the pipeline like Polymyxin B, Colistin (Polymyxin E), Rifampicin and Tigecycline, the judicious selection of antibiotics to treat MBL producing isolates should be implemented.

Fig.1 Number of P.aeruginosa and other isolates from different specimens
n= 580

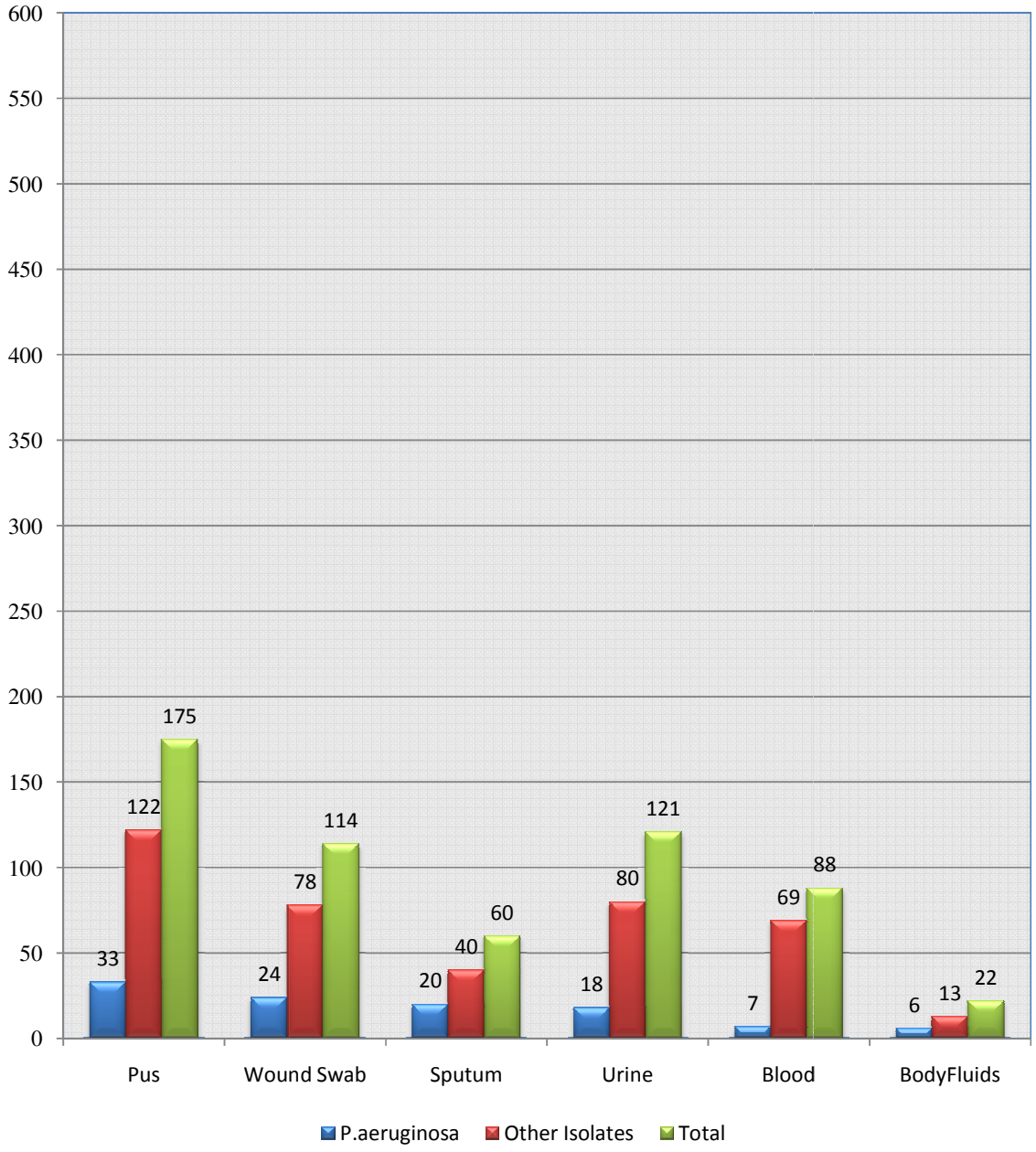


Fig. 2 Resistance pattern of P.aeruginosa isolates to various drugs

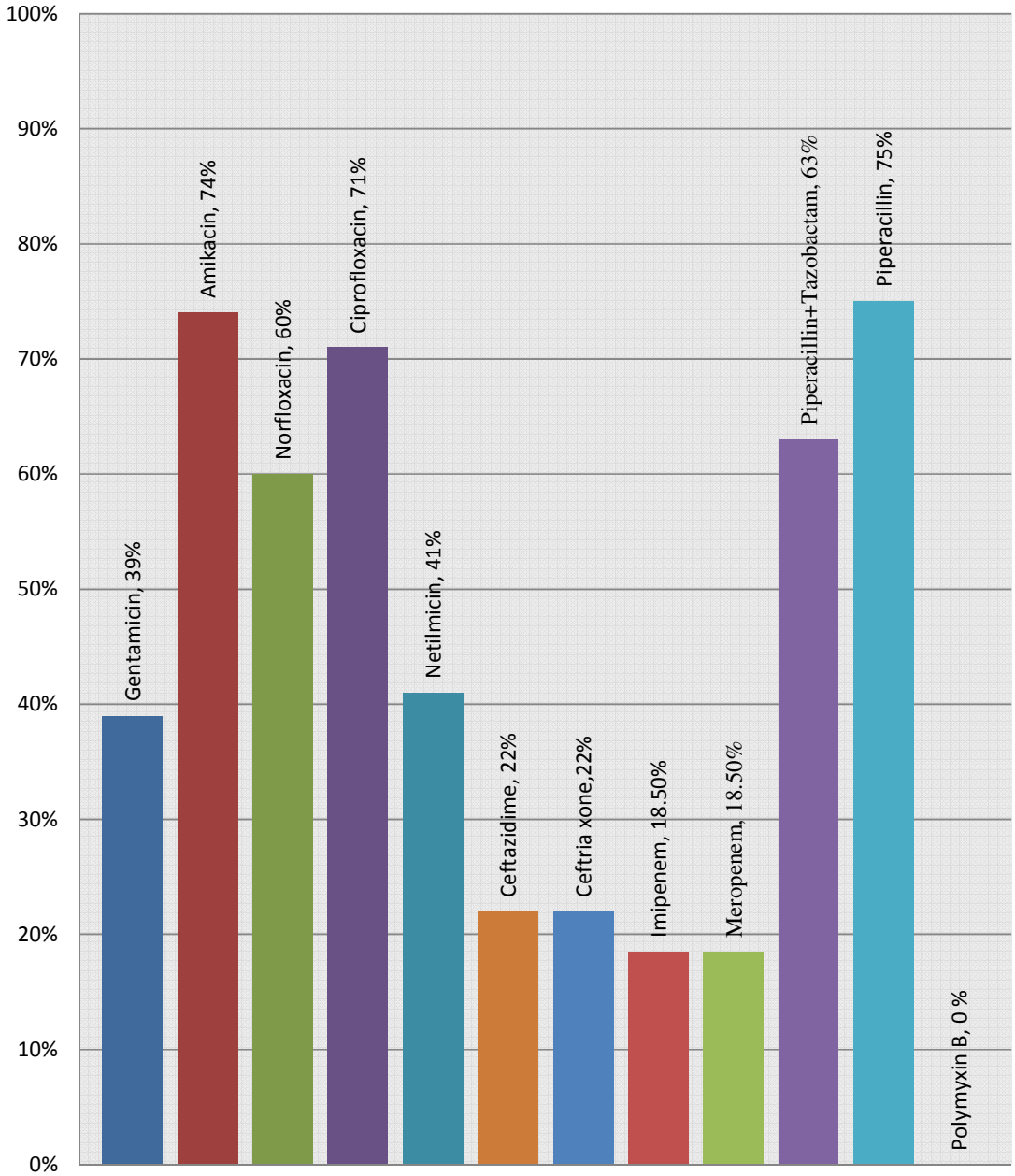


Fig.3 MIC determination by Agar Dilution method n=108

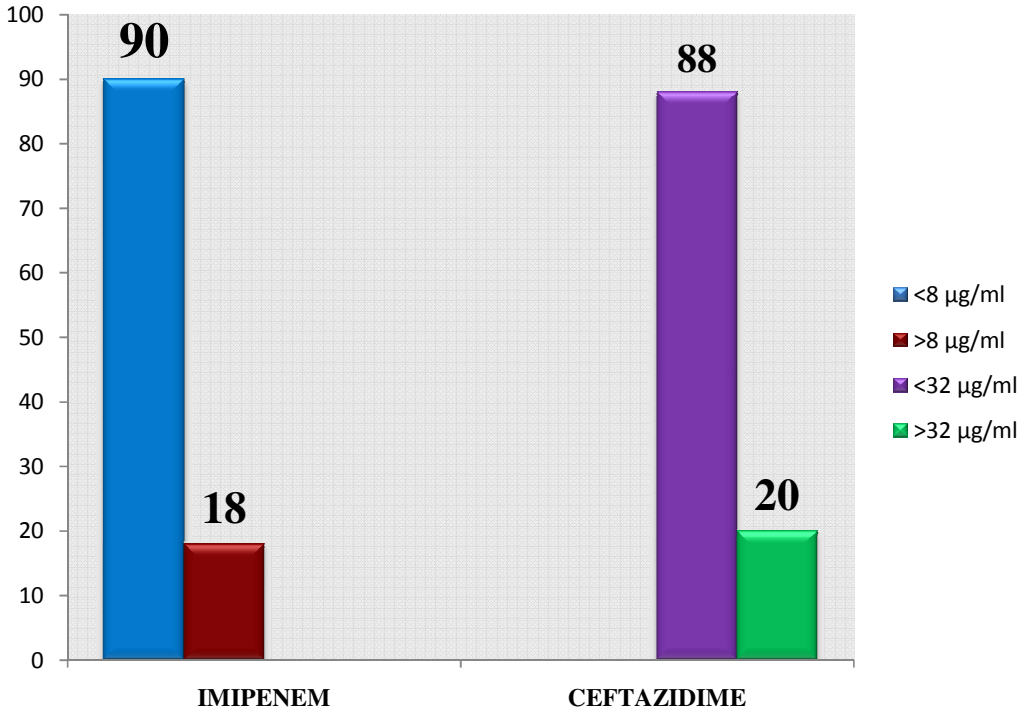


Fig.4 Isolates showing four fold reduction in MIC with EDTA

Fig.5 MBL detection by Phenotypic methods n = 20

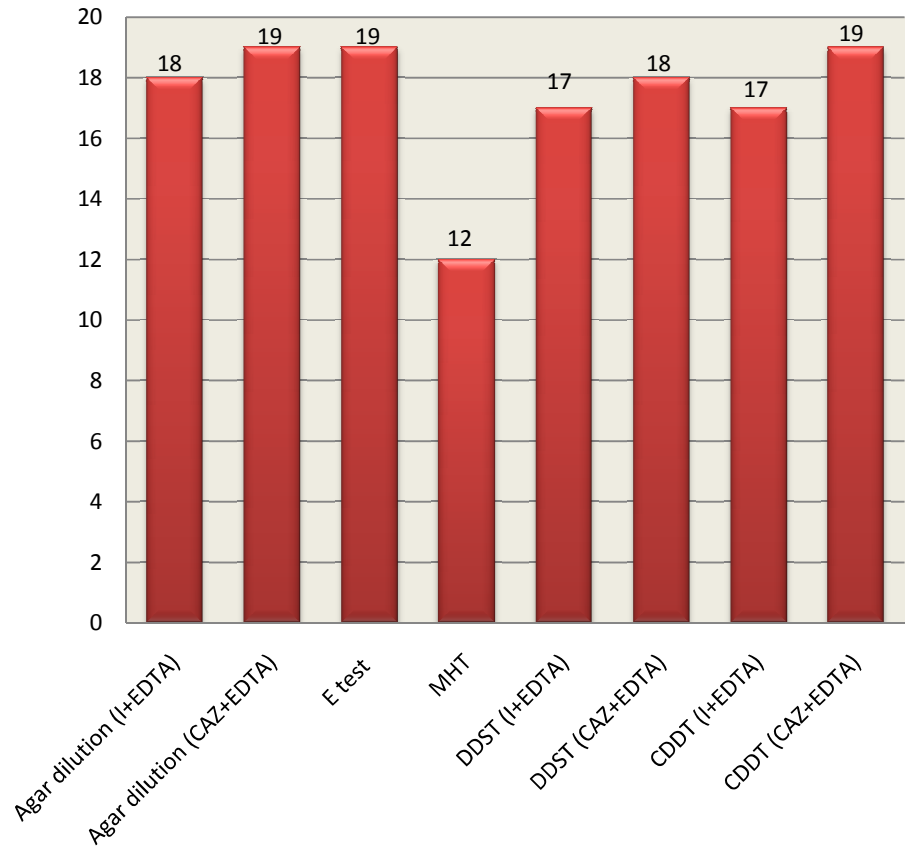


Fig.6 MBL genes detected by PCR n=20

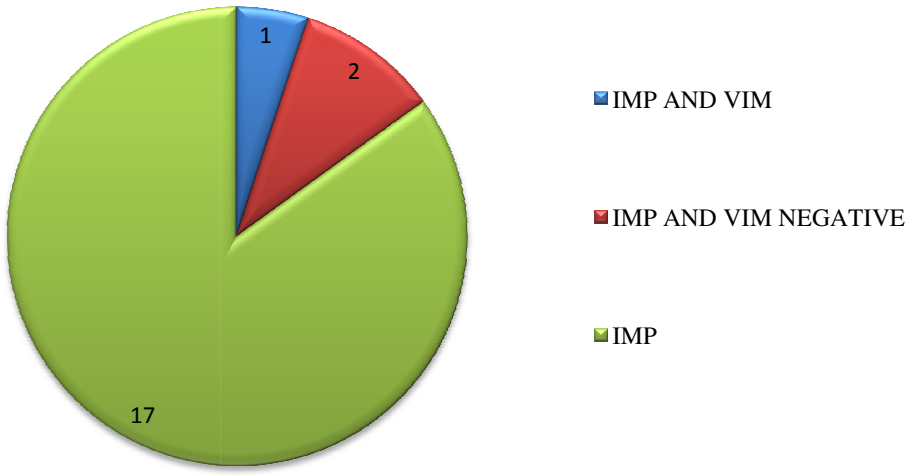


Fig.7 Age wise distribution of P.aeruginosa n=108

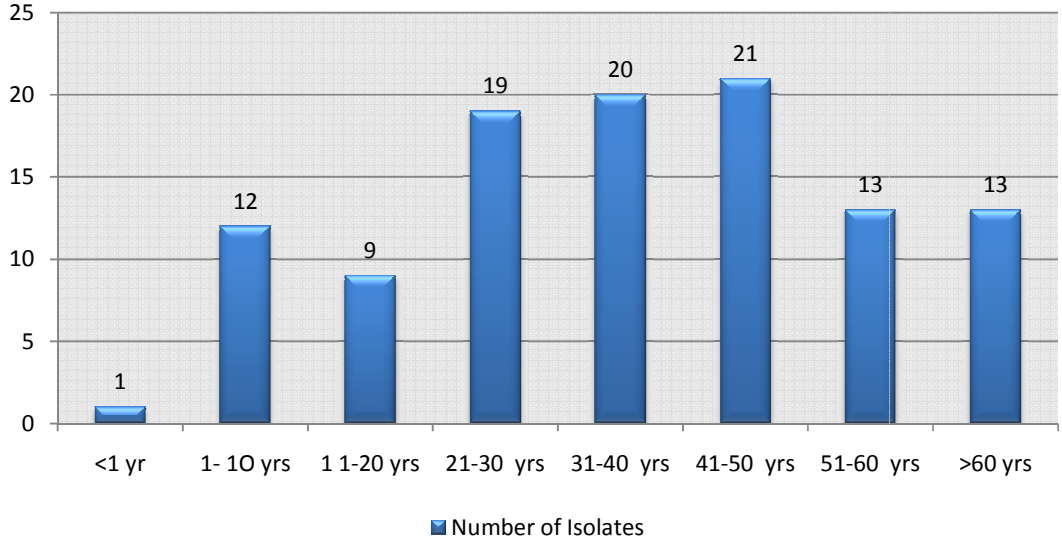


Fig.8 Age wise distribution of MBL producing P.aeruginosa n=18

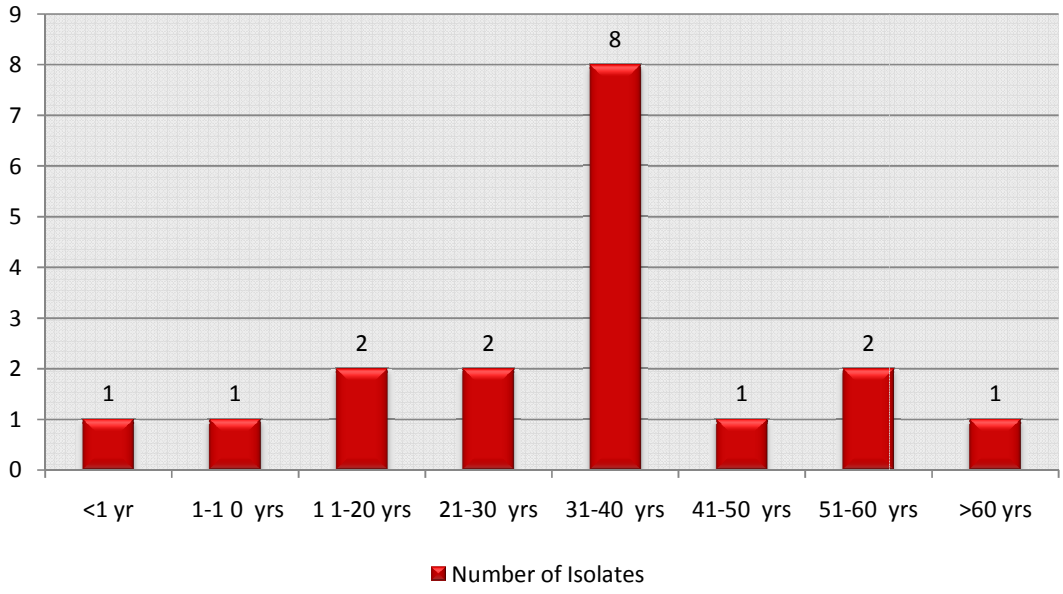


Fig.9 Sex distribution of P.aeruginosa n= 108

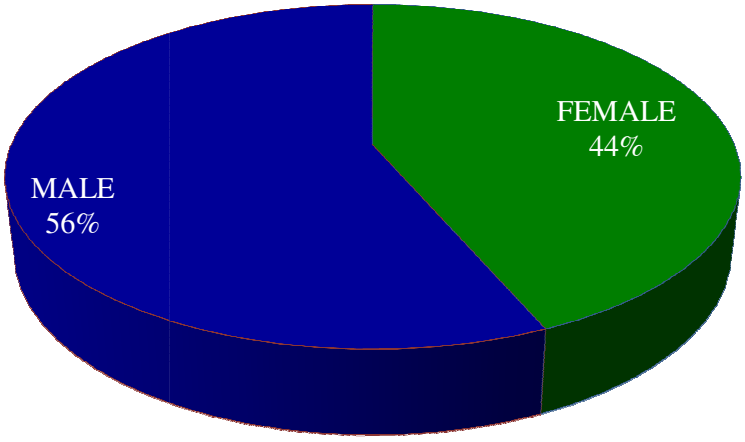


Fig.10 Sex distribution of MBL producing P.aeruginosa n=18

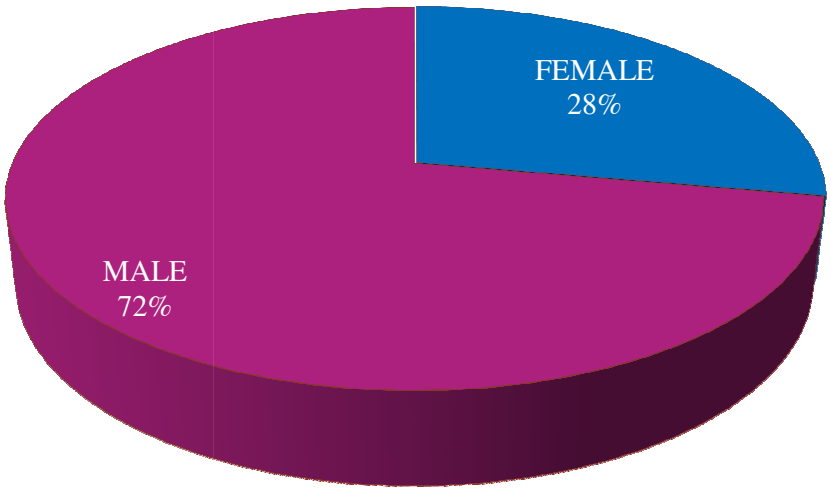


Fig.11 Specimen wise isolation of Pseudomonas aeruginosa n=108

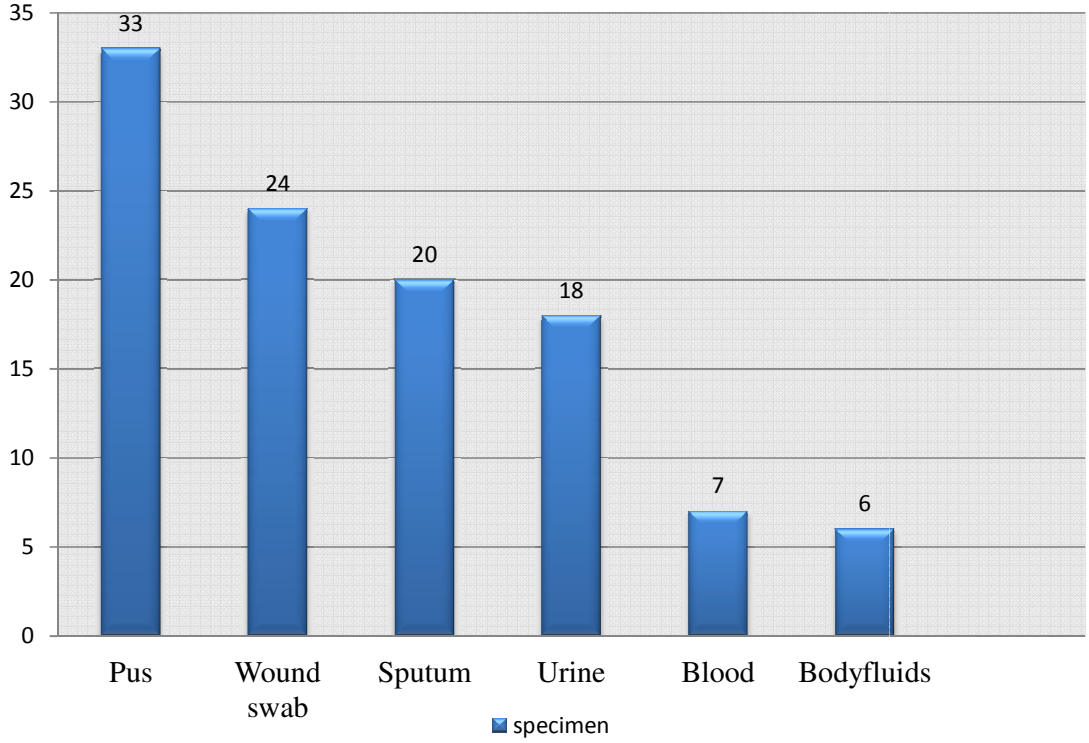


Fig.12 Specimen wise isolation of MBL producing Pseudomonas aeruginosa n =18

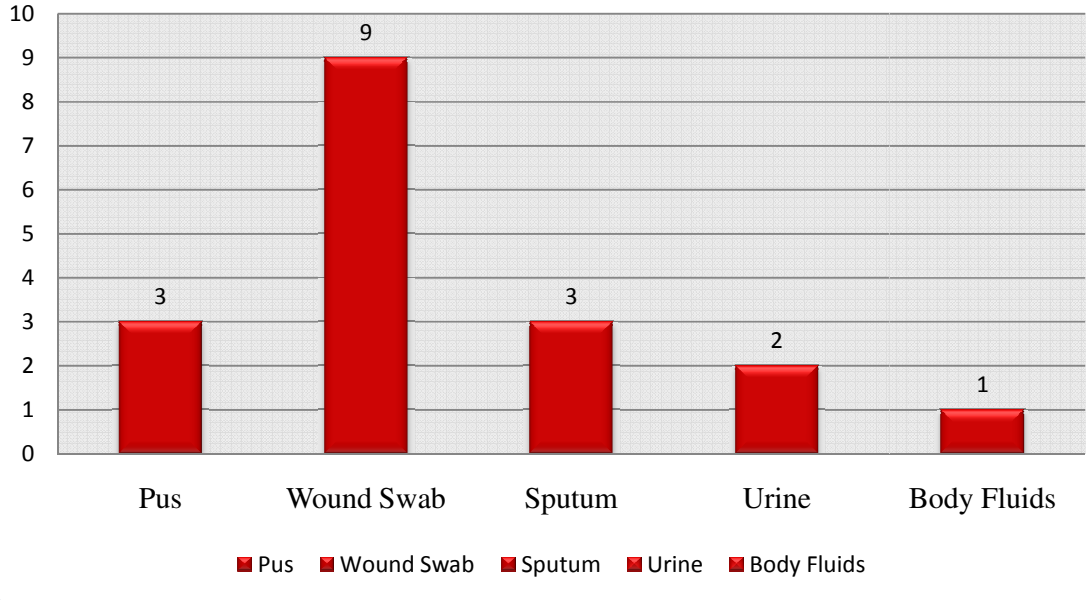


Fig.13 Risk factors associated with Pseudomonas aeruginosa n= 108

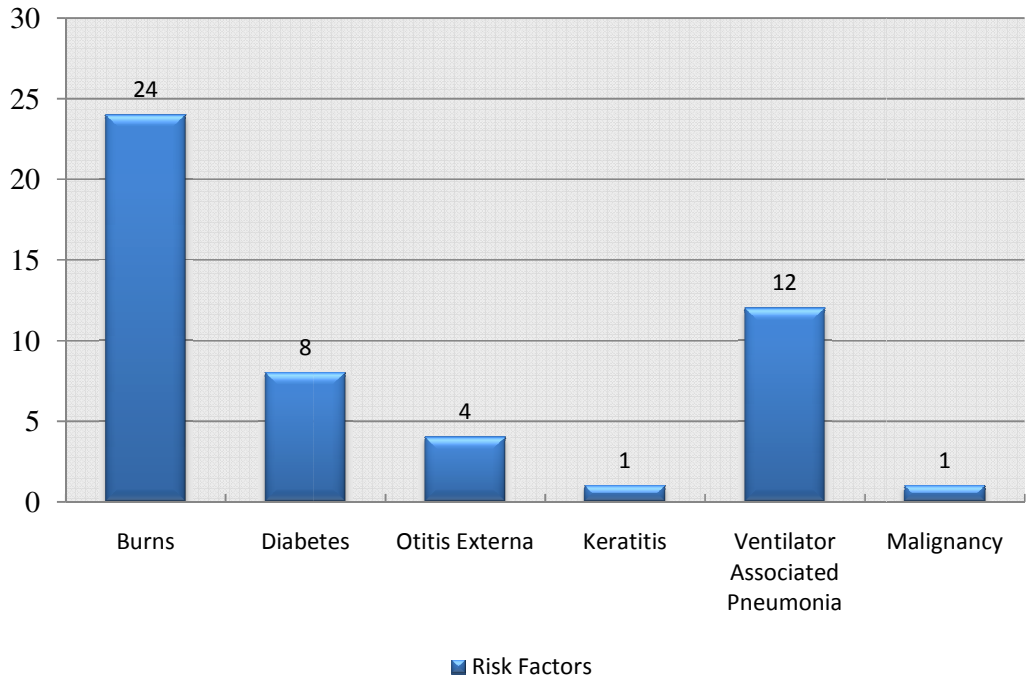


Fig.14 Risk factors associated with MBL producing P.aeruginosa n=18

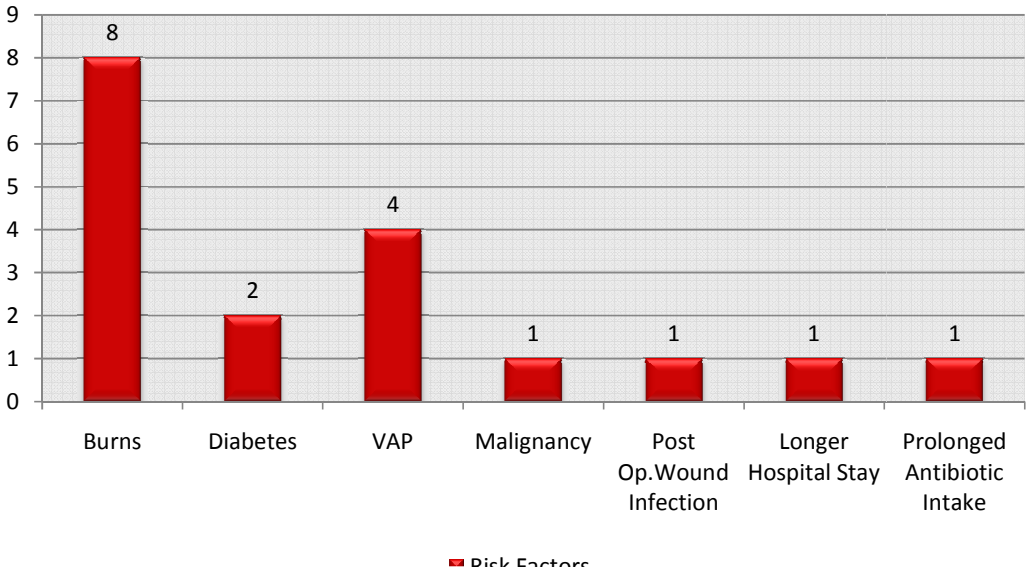
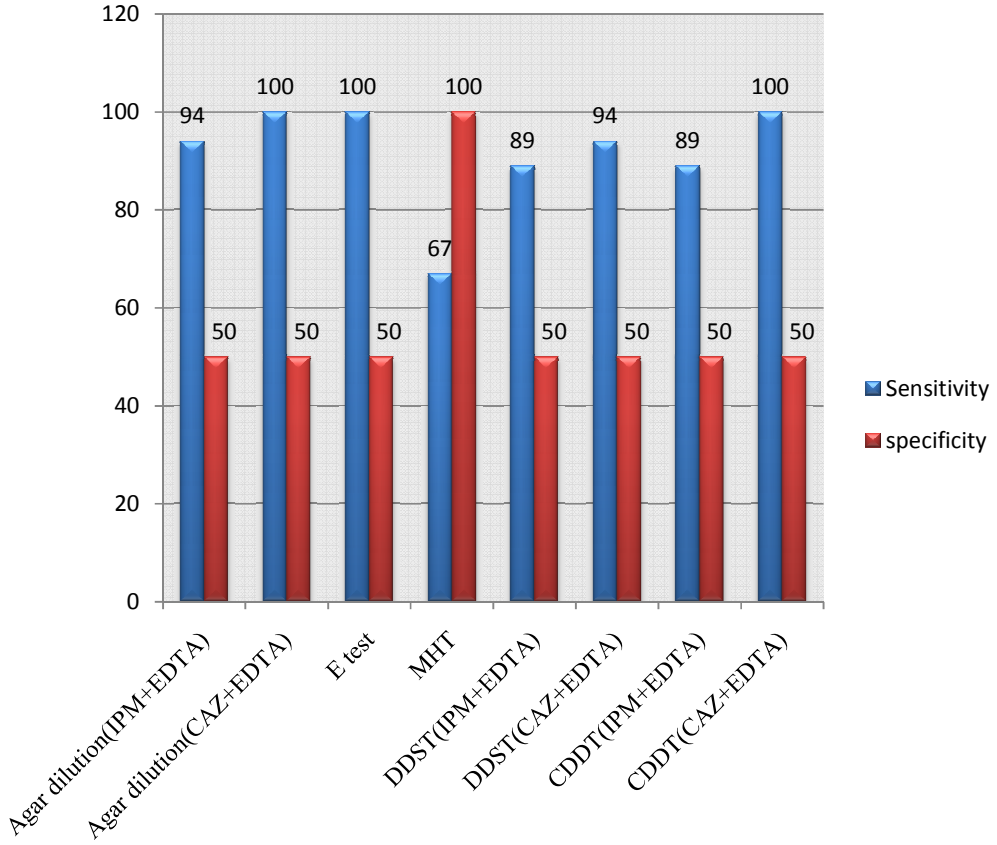


Fig.15 sensitivity & Specificity of various phenotypic methods



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ANNEXURE – 1

PREPARATION OF GRAM STAIN

GRAM STAIN REAGENTS

1. Methyl violet - Primary stain

   Methyl violet 10 g

   95% ethyl alcohol 100 ml

   Distilled water 1 L

2. Gram's Iodine – Mordant

   Iodine 10 g

   Potassium iodide 20g

   Distilled water 1 L

3. Acetone - Decolouriser

4. Dilute Carbol Fuchsin - Counter stain

   Basic fuchsin 0.3g

   95% Ethyl alcohol 10 ml

   Phenol crystals, melted 5 ml

   Distilled water 95 ml

Basic fuchsin was dissolved in alcohol.5 % phenol solution was added and was allowed to stand overnight. Then the solution filtered through coarse filter paper.

ANNEXURE - 2

PREPARATION OF MEDIA

PREPARATION OF NUTRIENT AGAR

Contents:

- Peptone – 5g
- Beef extract – 1.5 g
- Yeast extract – 1.5g
- Sodium chloride – 5g
- Agar – 15g

28 g of the contents were suspended in 1000 ml of distilled water. It was heated to boiling to dispense the medium completely. Medium was sterilised by autoclaving at 121 ˚ c at 15 lbs pressure for 15 minutes.

PREPARATION OF MAC KONKEY AGAR

Contents:

Sodium taurocholate 5.0 g

Peptone 20.0 gm

Sodium chloride 5.0 g

Lactose 10.0 g

Agar 15.0 g

Distilled water 1000 ml

Neutral red (2% solution in 50% ethanol) 3.5 ml

- 5 g sodium taurocholate or bile salts, 20 g of peptone, 5 g sodium chloride and 15 g agar were mixed with 1000 ml water.
- Steamed until the solids were dissolved.
- Cooled to about 50˚ C, and at this temperature the reaction was adjusted to pH 7.5 to 7.8. Autoclaved at 121˚C for 15 minutes and filtered while hot through a good grade of filter paper, or a plug of cotton wrapped in gauze placed in the funnel.
- The reaction of the filtrate was adjusted to pH 7.3 at 50˚C or pH 7.5 at room temperature. 10 gm lactose and 3.5 ml of 2% solution of neutral red in 50% ethanol were added. Mixed thoroughly and distributed in flasks and sterilized in the autoclave at 121˚C for 15 minutes.
- For use, melted in the steamer, poured into sterile petri dishes and allowed to set.

PREPARARION OF BLOOD AGAR

Nutrient agar 100 ml

Sheep blood (defibrinated) 10 ml

- The sterile nutrient agar was melted by steaming and cooled to 45˚C.
- 5% -10% sheep blood was added aseptically with constant shaking.
- The blood was mixed with molten nutrient agar thoroughly but gently, to avoid froth formation.To remove the bubbles,media was flamed.
- Immediately poured into petri dishes and allowed to set.

PREPARATION OF MUELLER – HINTON AGAR

Contents:

Beef extract 2.0 gm

Acidicase Peptone 17.5 gm

Starch 1.5 gm

Agar 17.0 gm

Distilled water 1000 ml

Final pH 7.4 + 0.2

Dissolved the ingredients in one liter of distilled water. Mixed thoroughly. Heated with frequent agitation and boiled for one minute. Dispensed and sterilized by autoclaving at 121˚C for 15 minutes. Should not be overheated. When remelting the sterile medium, heated as briefly as possible.

PREPARATION OF TSI MEDIUM:

Contents:

Beef extract 3g

Yeast extract 3g

Peptone 20g

Glucose 1g

Lactose 10g

Sucrose 10g

Ferric citrate 0.3g

Sodium chloride 5g

Sodium thiosulphate 0.3g

Agar 12g

Phenol red 0.2%

Distilled water 1 L

The solids were heated to dissolve and the indicator solution was added, mixed and tubed. Sterilized at 121˚c for 15 minutes and cooled to form slopes with deep (3 cm) butts.

PREPARATION OF CITRATE MEDIUM:

Contents:

Sodium chloride 5g

Magnesium sulphate 0.2g

Ammonium dihydrogen phosphate 1g

Sodium citrate 5g

Agar 20 g

Bromothmol blue 0.2% 40 ml

Distilled water 1 litre

The above contents were dispensed and autoclaved at 121˚c for 15 minutes and allowed to set as slopes.

PREPARATION OF OXIDATION-FERMENTATION MEDIUM:

Contents:

Peptone 2g

Sodium chloride 5g

Dipotassium hydrogen phosphate 0.3g

Bromothymol blue (1 % aqeous solution) 0.03g

Agar 3g

Distilled water 1000 ml

The pH was adjusted to 7.1 before adding the bromothymol blue and the medium was autoclaved in a flask at 121˚c for 15 minutes. The carbohydrate(Glucose) to be added was sterilized separately and added to give a final concentration of 1%.The medium was then tubed to a depth of about 4 cm.

PREPARATION OF NITRATE MEDIUM:

Contents:

Potassium nitrate 0.2g

Peptone 5g

Distilled water 1000ml

The above contents were mixed and tubed in 5 ml amounts and autoclaved at 121˚c for 15 minutes.

Test reagent:

Solution A: 8 g of sulphanilic acid was dissolved in 1 L of acetic acid 5 mol/litre

Solution B:5 g of alpha-naphthlamine in 1 L of acetic acid 5 mol/litre. Immediately before use,equal volumes of solutions A and B were mixed to get the test reagent.

PREPARATION OF ARGININE DIHYDROLASE TEST MEDIUM:

Contents:

Peptone 5g

Meat extract 5g

Glucose 0.5g

Pyridoxal 5mg

Bromocresol purple 5 mg (1 in 500 solution) 5ml

Cresol red (1 in 500 solution) 2.5 ml

Distilled water 1000 ml

The solids were dissolved in water and the pH adjusted to 6.0 before the addition of indicators .This is the basal medium and to it was added the amino acid 1% L-arginine hydrochloride .1 ml quantities were distributed in small tubes and autoclaved at 121˚c for 15 minutes. Sterile liquid paraffin was added to provide a layer about 5 mm thick above the medium.

ANNEXURE -3

DATA COLLECTION PROFORMA

1. Case No.

2. Name and address of the patient:

3. Age & sex:

4. OP & IP No:

5. Ward and Unit:

6. Date of admission:

7. Occupation and income:

8. Clinical diagnosis:

9. Relevant co-existing clinical conditions/illnesses:

10. History of presenting illness:

11. Past history:

12. Personal history:

13. Treatment History:

    (Antibiotics taken if any)

14. Sample collected:

15. Date of sample collection:

Ref. No. 00216 /E4/3/2011

Govt.Rajaji Hospital,Madurai.20.
Dated: 27.01.2012

Institutional Review Board / Independent Ethics Committee.

Dr. A. Edwin Joe, M.D (FM), BL.,
Dean, Madurai Medical College & 2521021 (Secy)
Govt Rajaji Hospital, Madurai 625020.
Convenor
grhethicssecy @gmail.com.

Sub: Establishment-Govt. Rajaji Hospital, aMadurai-20-
Ethics committee-Meeting Agenda-communicated-regarding.

The next Ethics Committee meeting of the Govt. Rajaji Hospital, Madurai was held at 11.00 Am to 1.00Pm on 27.01.2012 at the Dean Chamber, Govt. Rajaji Hospital, Madurai. The following members of the committee have been attended the meeting.

1. Dr.N.Vijayasankaran,M.ch(Uro.) 094-430-58793 0452-2584397	Sr.Consultant Urologist Madurai Kidney Centre, Sivagangai Road,Madurai	Chairman
2. Dr.P.K. Muthu Kumarasamy, M.D., 9843050911	Professor & H.O.D of Medical Oncology(Retired)	Member Secretary
3. Dr.T.Meena,MD 094-437-74875	Professor of Physiology, Madurai Medical College	Member
4. Dr. S. Thamilarasi, M.D (Pharmacol)	Professor of pharmacology	
5.Dr.Moses K.Daniel MD(Gen.Medicine) 098-421-56066	Professor of Medicine Madurai Medical College	Member
6.Dr.M.Gobinath,MS(Gen.Surgery) 097-871-50040	Professor of Surgery Madurai Medical College	Member
7.Dr.S. Dilshadh, MD(O&G)	Professor of OP&Gyn Madurai Medical College	Member
8.Dr.S.Vadivel Murugan., M.D, 097-871-50040	Professor of Medicine Madurai Medical College	Member
9.Shri.M.Sridher,B.sc.B.L. 099-949-07400	Advocate, 623-B.II.Floor,East II Cross, K.K.Nagar,Madurai.20.	Member
10.Shri.O.B.D.Bharat,B.sc., 094-437-14162	Businessman Plot No.588, K.K.Nagar,Madurai.20.	Member
11.Shri. S.sivakumar,M.A(Social) Mphil 093-444-84990	Sociologist, Plot No.51 F.F, K.K Nagar, Madurai.	Member

Following Projects were approved by the committee

Sl. No	Name of P.G.	Course	Name of the Project	Remarks
19.	Divya. B	M.D (microbiol)	Phenotypic and genotypic characterization of metallo-betalactamase production by Pseudomonas acruginosa.	Approved
20.	Thilakavathi.	PG, M.D (microbiol)	Phenotypic and genotypic discrimination between invasive and saprophytic coagulase negative Staphylococci strains.	Approved

Please note that the investigator should adhere the following: She/He should get a detailed informed consent from the patients/participants and maintain Confidentially.

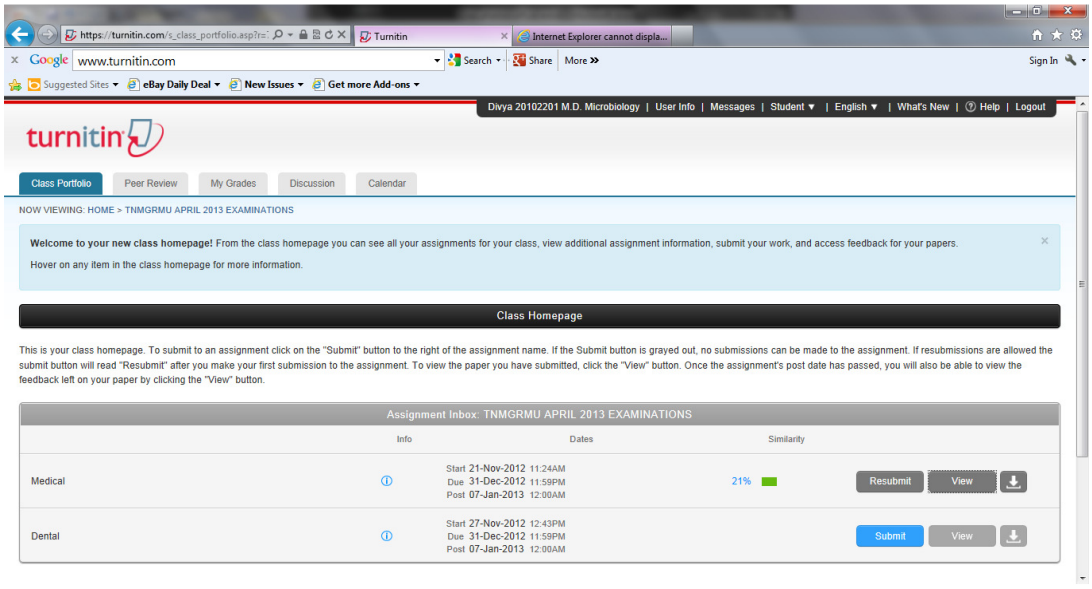
1. She/He should carry out the work without detrimental to regular activities as well as without extra expenditure to the institution to Government.
2. She/He should inform the institution Ethical Committee in case of any change of study procedure site and investigation or guide.
3. She/He should not deviate for the area of the work for which applied for Ethical clearance.
She/He should inform the IEC immediately, in case of any adverse events pr Serious adverse reactions.
4. She/he should abide to the rules and regulations of the institution.
5. She/He should complete the work within the specific period and apply for if any Extension of time is required She should apply for permission again and do the work.
6. She/He should submit the summary of the work to the Ethical Committee on Completion of the work.
7. She/He should not claim any funds from the institution while doing the word or on completion.
8.She/He should understand that the members of IEC have the right to monitor the work with prior intimation.

22/2

DEAN

To
All the above members and Head of the Departments concerned.
All the Applicants.

22/2/12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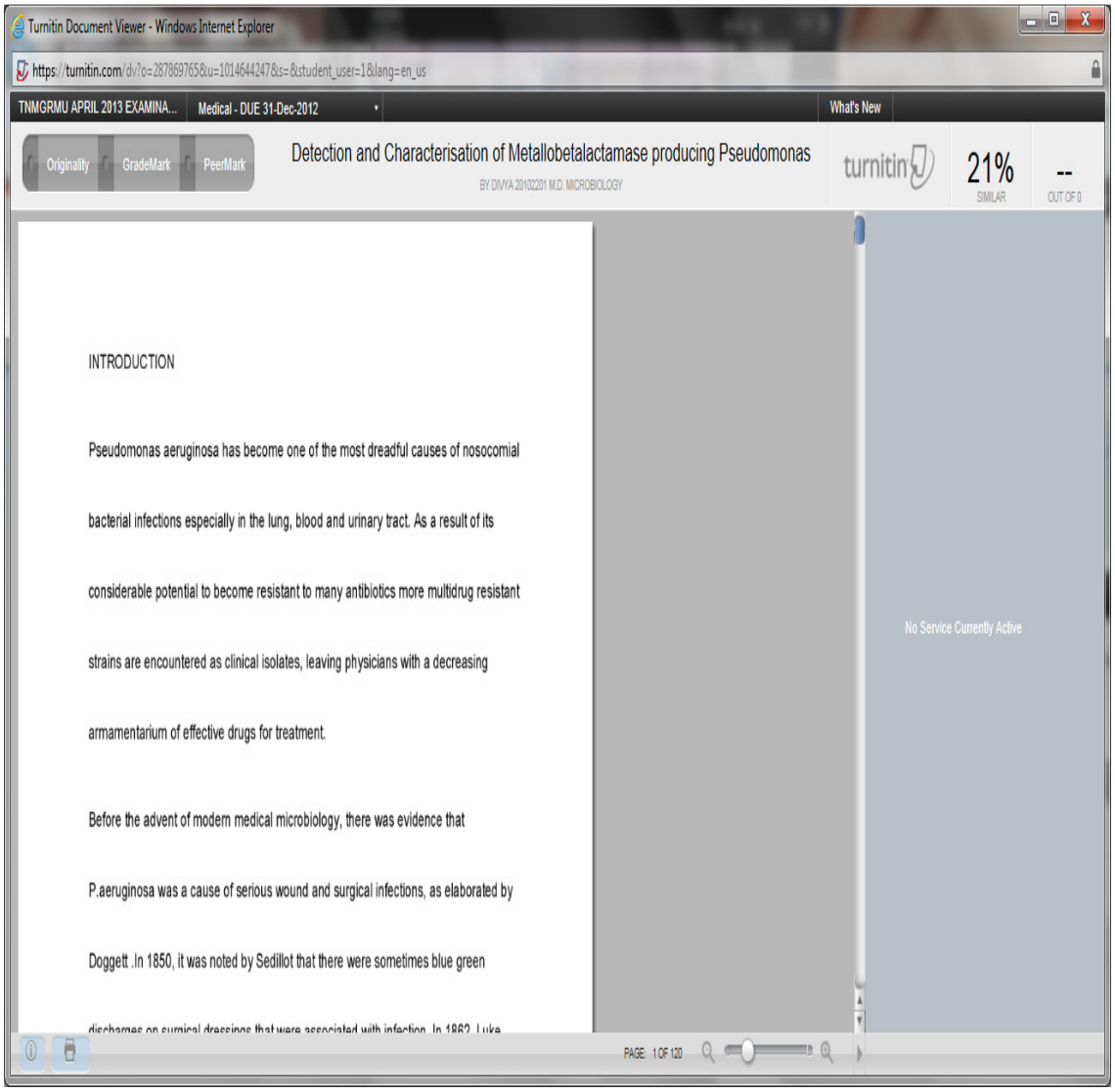
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Detection and Characterisation of Metallobetalactamase producing Pseudomonas
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INTRODUCTION
Pseudomonas aeruginosa has become one of the most dreadful causes of nosocomial bacterial infections especially in the lung, blood and urinary tract. As a result of its considerable potential to become resistant to many antibiotics more multidrug resistant strains are encountered as clinical isolates, leaving physicians with a decreasing armamentarium of effective drugs for treatment.
Before the advent of modern medical microbiology, there was evidence that P.aeruginosa was a cause of serious wound and surgical infections, as elaborated by Doggett .In 1850, it was noted by Sedillot that there were sometimes blue green
No Service Currently Active
PAGE: 1 OF 120

FIG. 1 NUTRIENT AGAR PLATE SHOWING GREEN PIGMENTED COLONIES

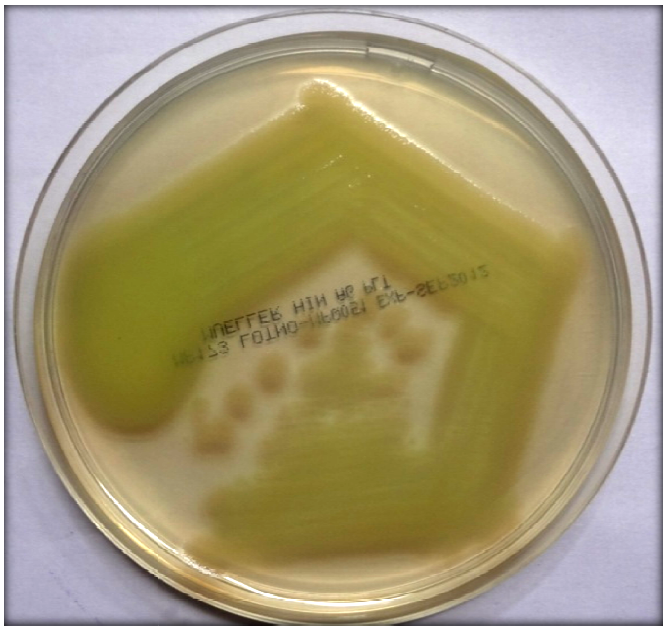


FIG.2 MACKONKEY AGAR PLATE SHOWING NON-LACTOSE FERMENTING COLONIES

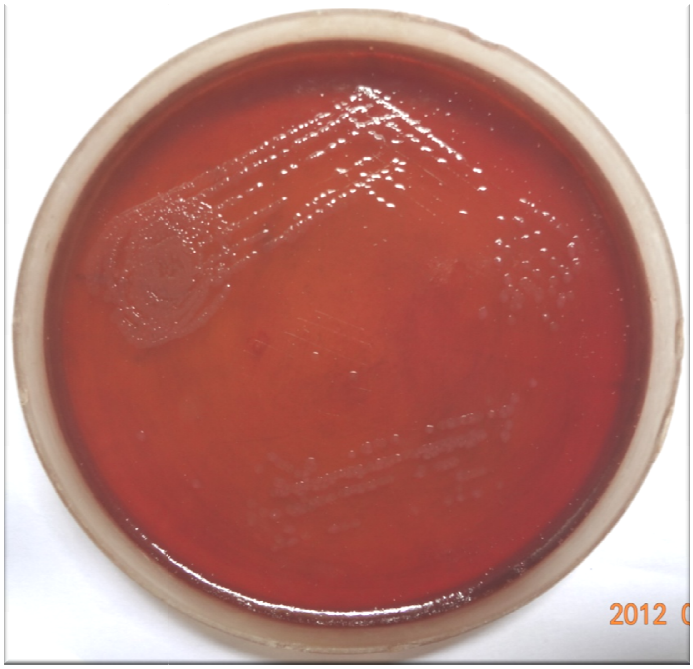
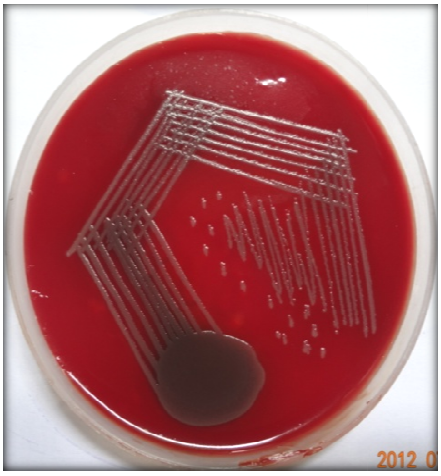


FIG.3 BLOOD AGAR PLATE SHOWING METALLIC SHEEN AND BETA HEMOLYTIC COLONIES



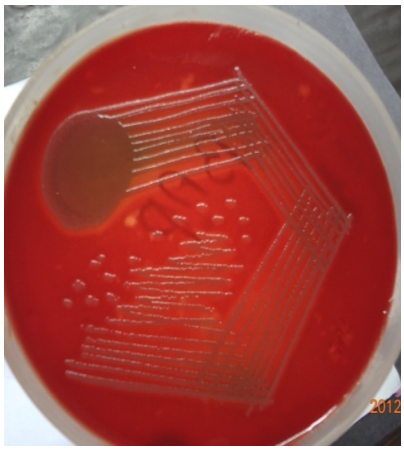


FIG.4 GRAM NEGATIVE BACILLI IN DISCRETE PATTERN

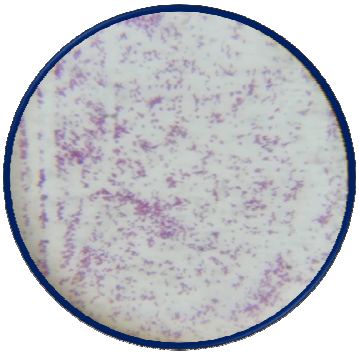


FIG.5 OXIDASE TEST

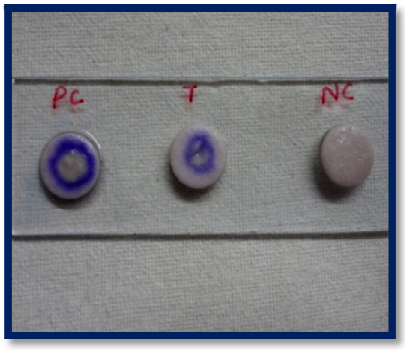


FIG.6 CATALASE TEST

FIG.7 BIOCHEMICAL REACTIONS

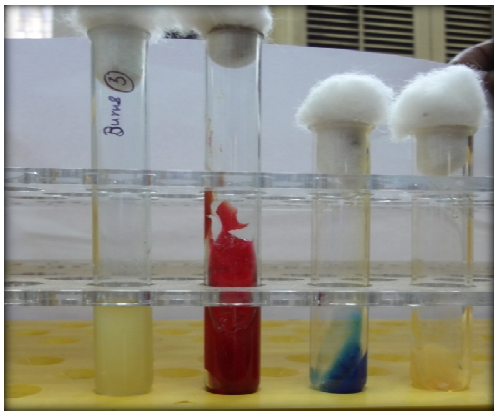


FIG.8 O/F TEST

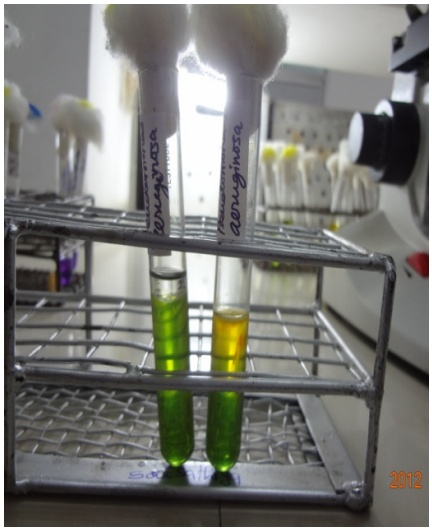


FIG.9 NITRATE REDUCTION TEST

FIG.10 ARGININE DIHYDROLASE TEST

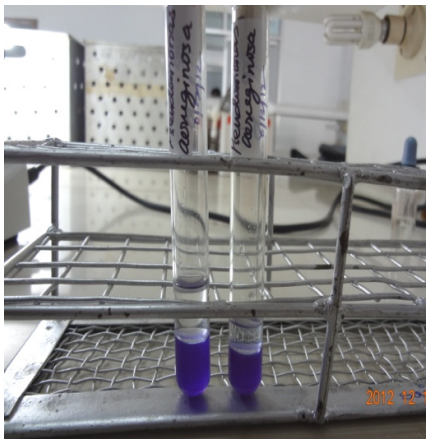


FIG.11 GELATIN LIQUEFACTION TEST

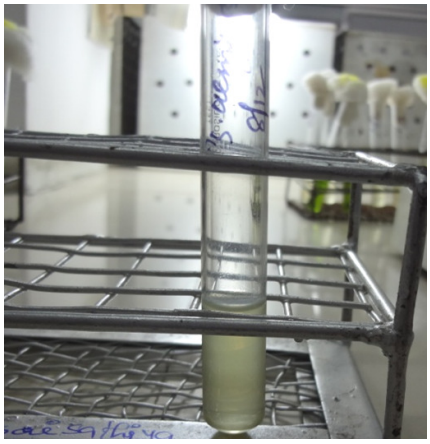
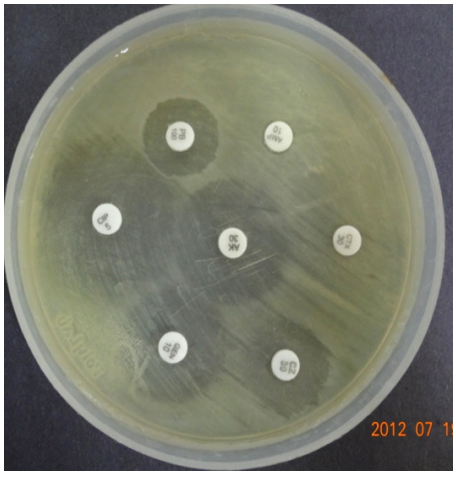
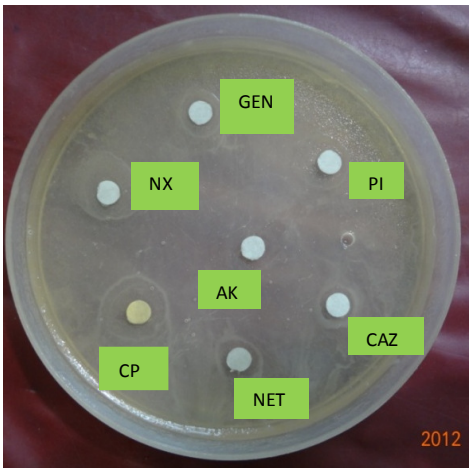


FIG.12 ANTIBIOGRAM OF P.AERUGINOSA





MULTIDRUG RESISTANCE

FIG.13 MEROPENEM SUSCEPTIBLE - ATCC P.AERUGINOSA

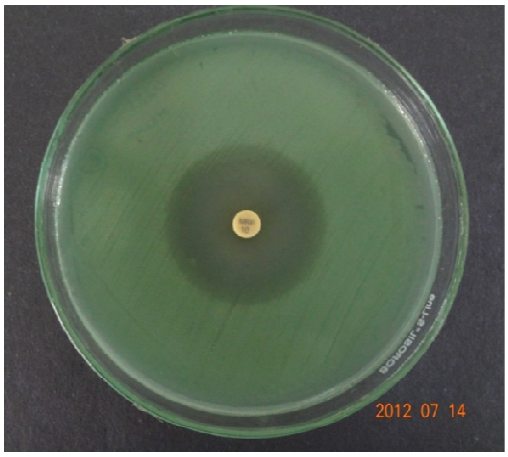


FIG.14 MEROPENEM RESISTANCE

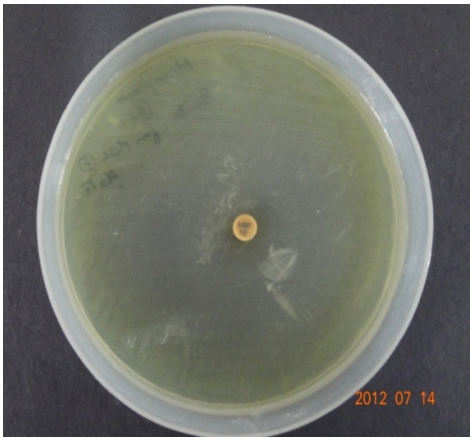
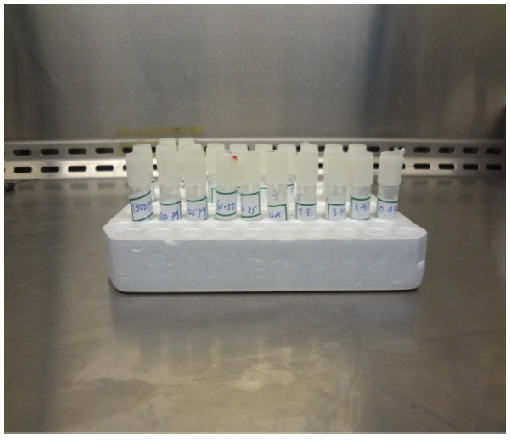


FIG.15 MIC DETERMINATION-AGAR DILUTION METHOD



DRUG DILUTION

MHA PLATES WITH IMIPENEM AND CEFTAZIDIME WITH SPOT INOCULATION

FIG.16 DRUG CONTROL

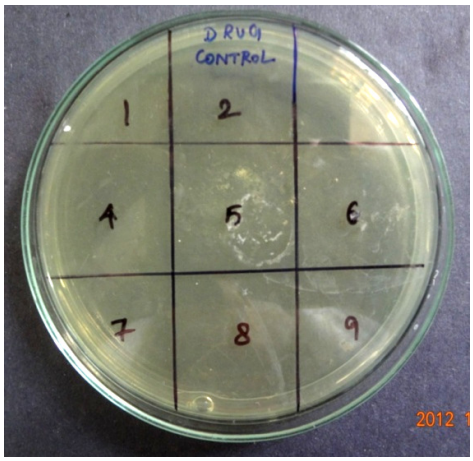


FIG.17 IMIPENEM RESISTANCE-

MIC 16μG

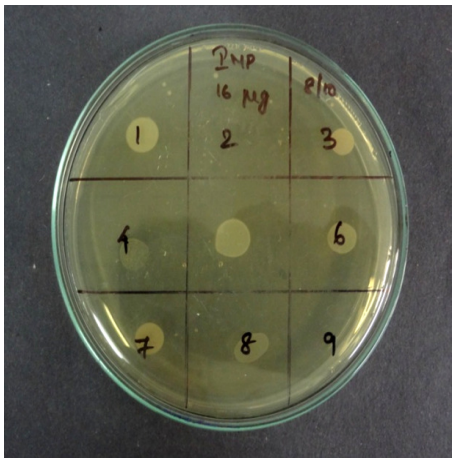


FIG.18 CEFTAZIDIME RESISTANCE-

MIC-1024 μG

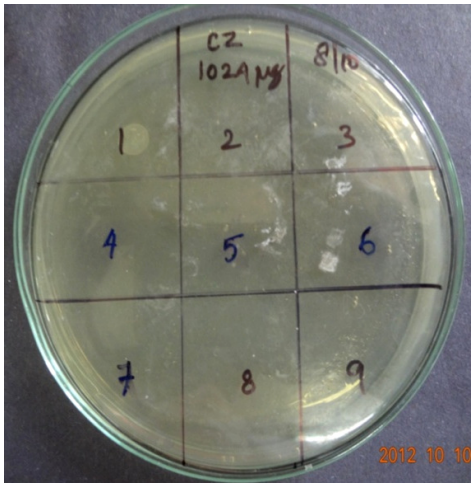


FIG.19 CEFTAZIDIME RESISTANCE-

MIC - 512 μG

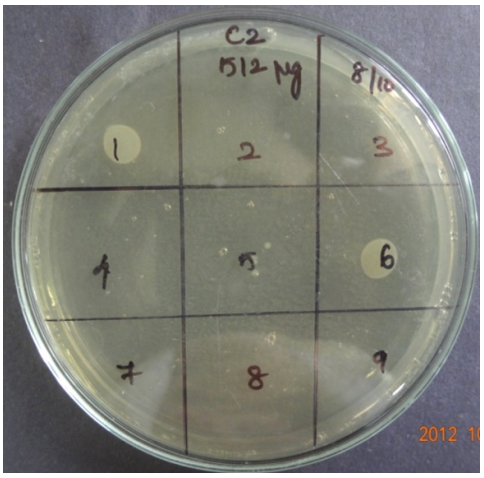
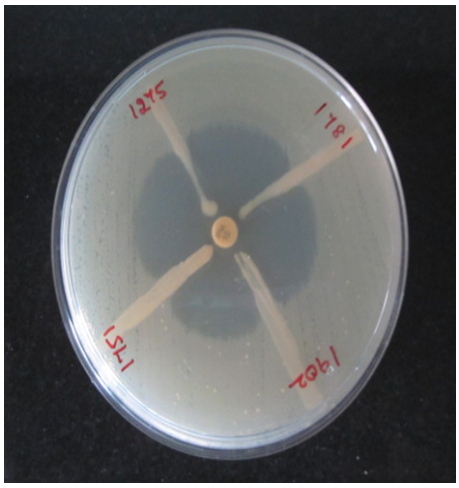


FIG20. MODIFIED HODGE TEST – MBL PRODUCER



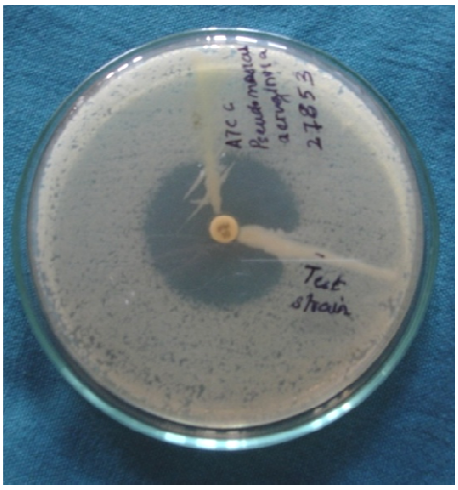
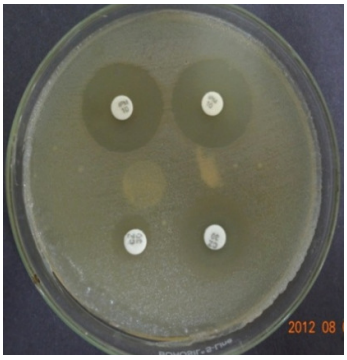


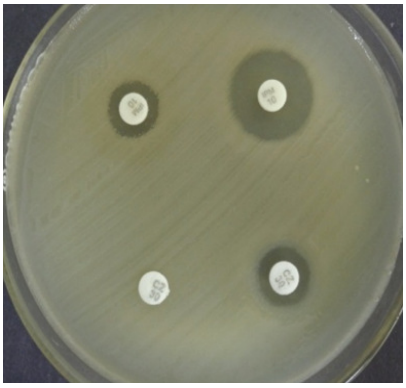
FIG.21 COMBINED DISC DIFFUSION TEST – IMIPENEM AND CEFTAZIDIME WITH EDTA

NON-MBL PRODUCER



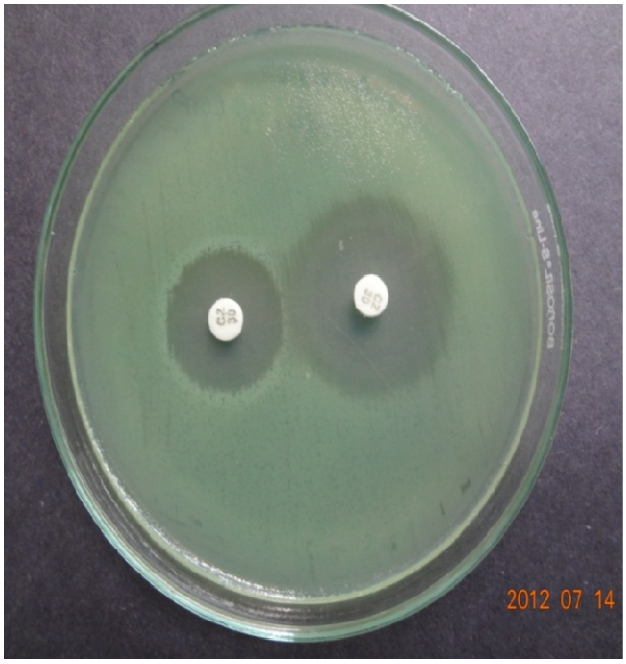
IPM, IPM+ EDTA - NO DIFFERENCE

MBL PRODUCER



IPM, IPM+EDTA > 5 MM DIFFERENCE

FIG.22 COMBINED DISC DIFFUSION TEST- CEFTAZIDIME WITH EDTA MBL PRODUCER



CAZ, CAZ+EDTA > 5 MM DIFFERENCE

FIG.23 DOUBLE DISC SYNERGY TEST- IMIPENEM WITH EDTA MBL PRODUCER

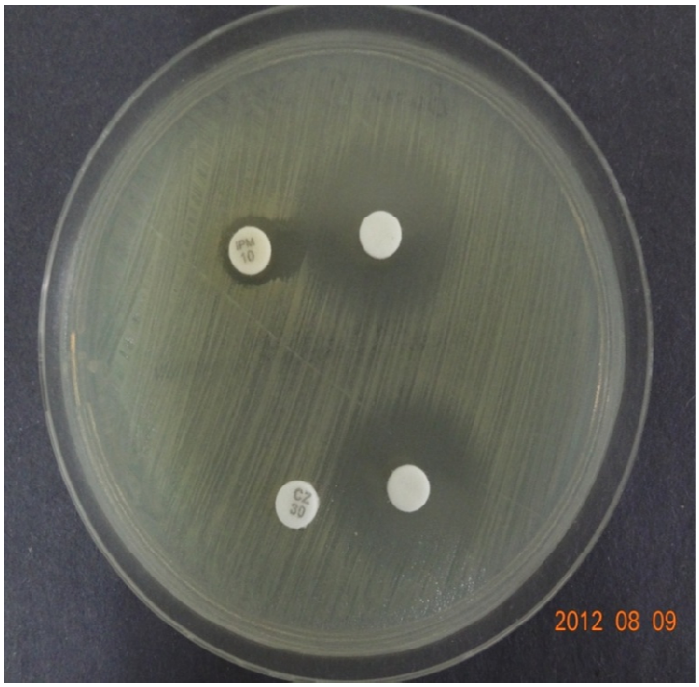
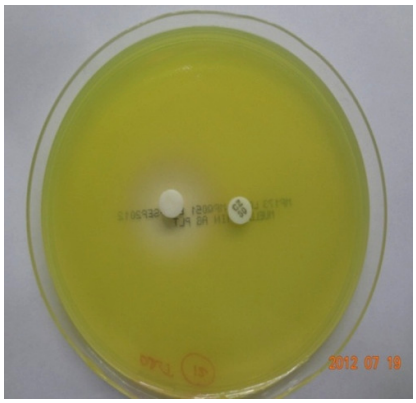


FIG.24 DOUBLE DISC SYNERGY TEST- CEFTAZIDIME WITH EDTA

NON-MBL PRODUCER



MBL PRODUCER

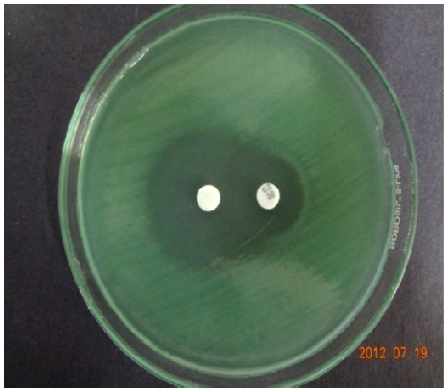
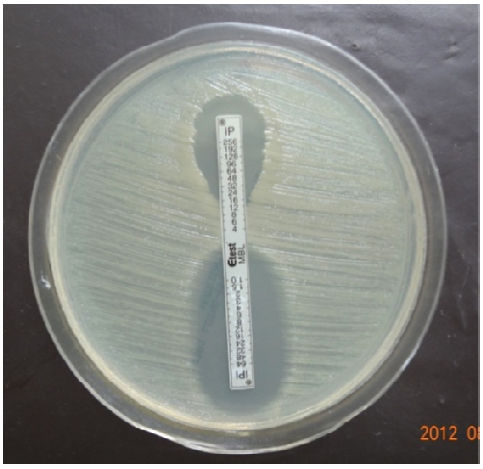


FIG.25 E TEST

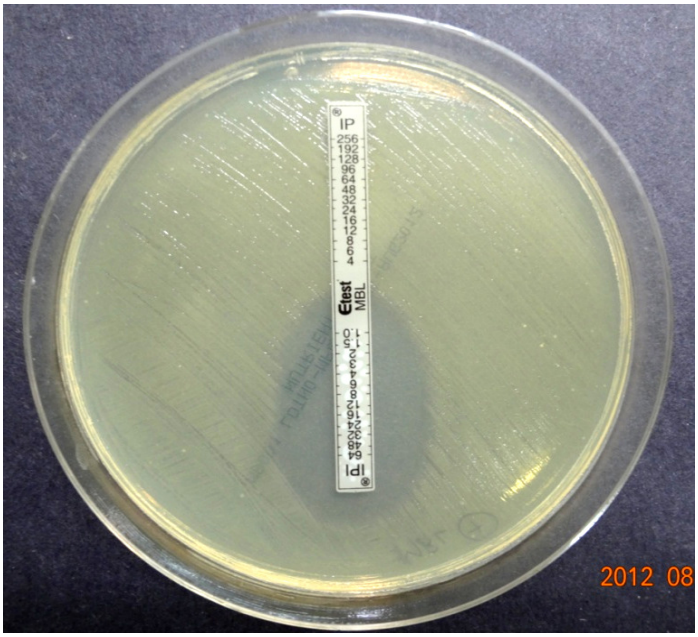
NON-MBL PRODUCER

MBL-PRODUCER



IPM - MIC- 16 µG, WITH EDTA <1 µG

FIG.25 E TEST - MBL PRODUCER

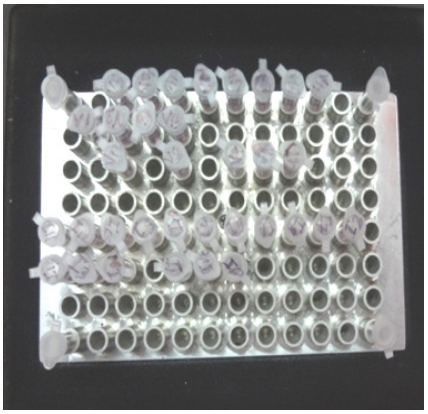


IMP MIC-256 µG, WITH EDTA <1 µG

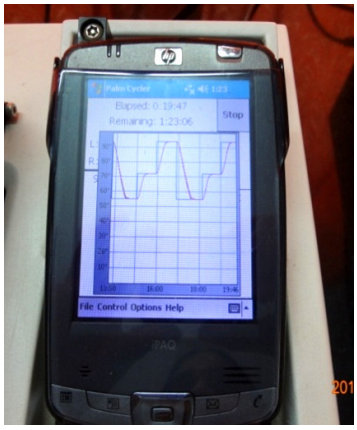
FIG. 26 MOLECULAR CHARACTERISATION- PCR

THERMOCYCLER

SAMPLES LOADED



PALM CYCLER



GEL ELECTROPHORESIS

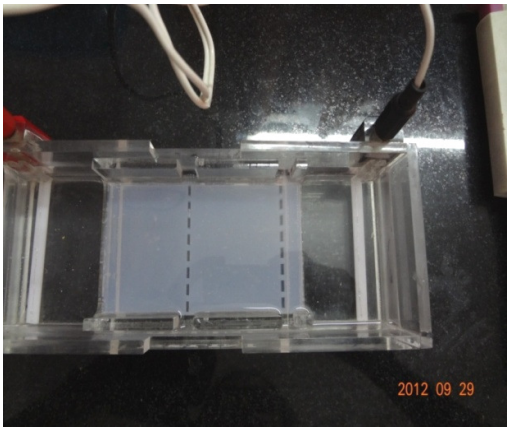
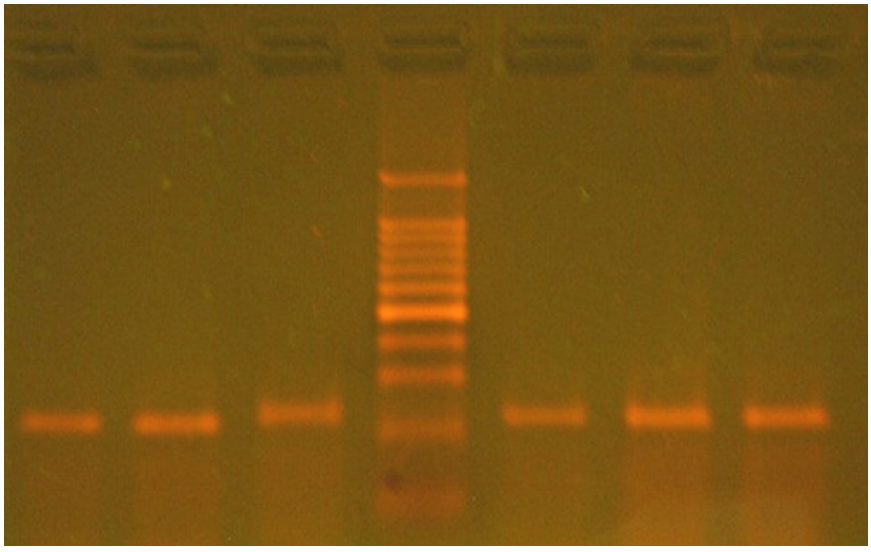
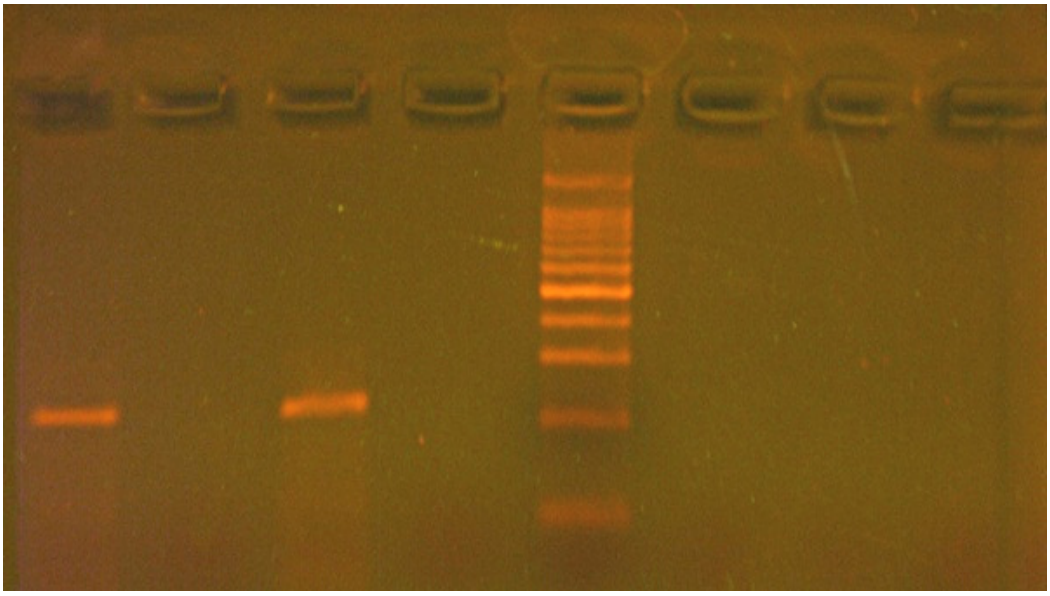


FIG.27 PCR GEL DOCUMENTATION PICTURE-IMP GENE



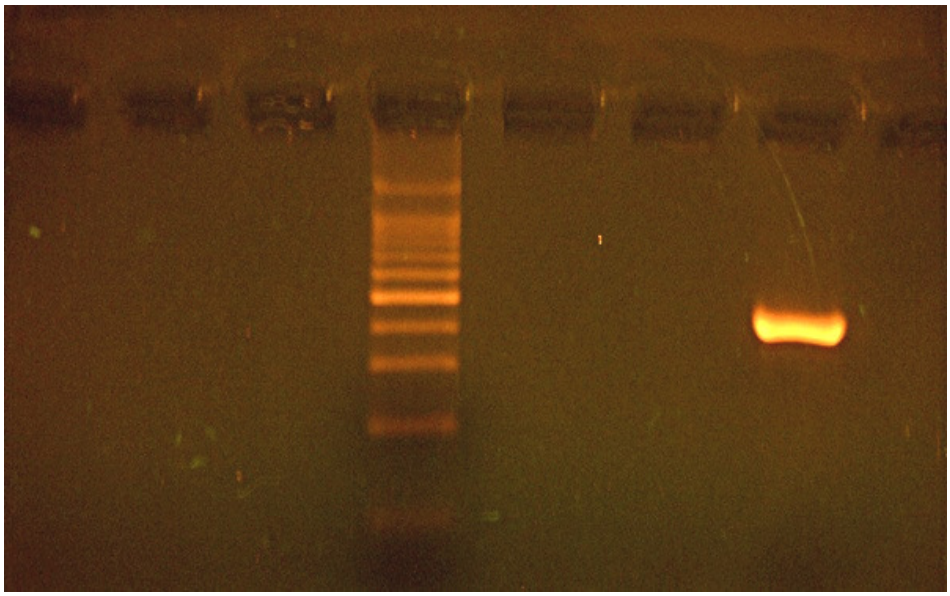
PC T1 T2 LADDER T3 T4 T5

FIG.27 PCR GEL DOCUMENTATION PICTURE-IMP GENE



PC NC TEST DNA LADDER

FIG.28 PCR GEL DOCUMENTATION PICTURE-VIM GENE



DNA LADDER TEST

MASTER CHART																												
S.NO	MICRO NO	AGE/SEX	DIAGNOSIS	SPECIMEN	ANTIBIOGRAM												AGAR DILUTION (MIC) *µg/ml*				MHT	DDST		CDDT		E TEST	PCR (IMP/VIM GENE)
					Piperacillin(75µg)	Piperacillin-tazobactam(100/10µg)	Ceftazidime(30µg)	Ceftriaxone(30µg)	Imipenem(10µg)	Meropenem(10µg)	PolymyxinB(300U)	Gentamicin(10µg)	Amikacin(30µg)	Norfloxacin(10µg)	Ciprofloxacin(5µg)	Netilmicin(30µg)	IMP	IMP+EDTA	CAZ	CAZ+EDTA		IMP+EDTA	CAZ+EDTA	IMP+EDTA	CAZ+EDTA		
1	2972	7/Fch	UTI	Urine	R	R	S	S	S	S	S	S	S	S	S	S	0.25		0.5								
2	2970	10/Fch	UTI	Urine	R	R	S	S	S	S	S	S	R	S	S	S	0.25		1								
3	624	16/F	Pne	Sput	R	R	S	S	S	S	S	S	S	S	S	S	0.5		2								
4	1516	48/M	WI	Pus	R	S	S	S	S	S	S	S	S	S	S	S	0.25		8								
5	1553	42/F	Abscess	Pus	R	R	S	S	S	S	S	S	R	S	S	S	0.25		0.5								
6	3790	23/F	PUO	Blood	S	R	S	S	S	S	S	S	S	S	S	S	1		1								
7	3821	14/F	PUO	Blood	S	R	S	S	S	S	S	S	R	S	S	S	0.25		4								
8	1669	63/M	CA rect	Pus	S	S	S	S	S	S	S	S	R	S	S	S	0.25		1								
9	684	28/F	Pne	Sputum	R	R	S	S	S	S	S	S	S	S	S	S	0.25		2								
10	1723	31/M	WI	Pus	R	R	S	S	S	S	S	S	S	S	S	S	0.25		1								
11	1706	70/M	keratitis	Eyesw ab	S	S	S	S	S	S	S	S	S	S	S	S	0.25		0.5								
12	604	28/F	WI	Pus	R	S	S	S	S	S	S	S	S	S	S	S	0.25		1								
13	1762	40/M	Pyothorax	Pus	S	S	S	S	S	S	S	S	S	S	S	S	0.25		0.5								
14	718	30/F	COPD	Sputum	R	R	S	S	S	S	S	S	S	S	S	S	0.25		2								
15	4129	57/M	Sepsis	Blood	R	R	S	S	S	S	S	S	R	S	S	S	0.25		4								
16	943	40/M	Burns	WS	R	R	R	R	R	R	S	R	R	R	R	R	16	0.5	128	8	P	P	P	P	P	P	I

S.NO	MICRO NO	AGE/SEX	DIAGNOSIS	SPECIMEN	ANTIBIOGRAM												AGAR DILUTION (MIC)				MHT	DDST		CDDT		E TEST	PCR (IMP/VIM GENE)
					Piperacillin(75µg)	Piperacillin-tazobactam(100/10µg)	Ceftazidime(30µg)	Ceftriaxone(30µg)	Imipenem(10µg)	Meropenem(10µg)	PolymyxinB(300U)	Gentamicin(10µg)	Amikacin(30µg)	Norfloxacin(10µg)	Ciprofloxacin(5µg)	Netilmicin(30µg)	IMP	IMP+EDTA	CAZ	CAZ+EDTA		IMP+EDTA	CAZ+EDTA	IMP+EDTA	CAZ+EDTA		
17	767	25/F	Pneumonia	Sputum	R	R	S	S	S	S	S	S	R	S	S	S	0.25		4								
18	781	57/M	PT defaulter	Sputum	S	S	S	S	S	S	S	S	R	S	S	S	0.25		2								
19	862	70/F	COPD	Sputum	R	R	S	S	S	S	S	S	S	S	S	S	0.25		4								
20	2144	35/F	SSI	Pus	S	S	S	S	S	S	S	S	R	S	S	S	0.25		0.5								
21	2141	50/M	# BB Leg	Pus	R	S	S	S	S	S	S	S	S	S	S	S	0.25		2								
22	940	31/M	Burns	WS	R	R	R	R	R	R	S	R	R	R	R	R	16	1	256	8	P	N	P	P	P	P	I
23	2138	48/M	venous ulcer	Pus	R	S	S	S	S	S	S	S	R	S	R	S	0.25		2								
24	4729	20/M	PUO	Blood	R	R	R	R	R	R	S	R	R	S	S	S	2		4								
25	867	60/F	COPD	Sputum	S	S	S	S	S	S	S	S	R	S	R	S	0.25		2								
26	206	38/M	Bronchiectasis	BAL	R	S	S	S	S	S	S	S	S	S	S	S	0.25		1								
27	2270	70/M	SSI	Pus	R	R	S	S	S	S	S	S	R	S	R	S	0.5		2								
28	886	38/F	Burns	WS	R	R	S	S	S	S	S	S	S	S	R	S	0.25		2								
29	991	30/F	Pne	Sputum	R	R	S	S	S	S	S	S	R	S	R	S	1		8								
30	746	30/M	Burns	WS	R	R	S	S	S	S	S	S	R	S	R	S	0.25		4								
31	5021	3/Fch	UTI	Urine	R	S	S	S	S	S	S	S	R	S	R	S	0.25		0.5								
32	2446	30/F	O.E	Pus	R	R	S	S	S	S	S	S	R	S	R	S	0.25		0.5								
33	929	18/M	Burns	WS	R	R	R	R	R	R	S	R	R	R	R	R	16	1	256	4	P	P	N	P	P	P	I
34	1145	55/M	Pne	Sputum	R	R	S	S	S	S	S	S	R	S	S	S	0.25		1								
35	1147	60/M	COPD	Sputum	R	S	S	S	S	S	S	S	S	S	S	S	0.5		2								
36	6619	35/F	UTI	Urine	R	R	S	S	S	S	S	S	R	S	S	S	0.25		4								

S.NO	MICRO NO	AGE/SEX	DIAGNOSIS	SPECIMEN	ANTIBIOGRAM												AGAR DILUTION (MIC)				MHT	DDST		CDDT		E TEST	PCR (IMP/VIM GENE)
					Piperacillin(75µg)	Piperacillin-tazobactam(100/10µg)	Ceftazidime(30µg)	Ceftriaxone(30µg)	Imipenem(10µg)	Meropenem(10µg)	PolymyxinB(300U)	Gentamicin(10µg)	Amikacin(30µg)	Norfloxacin(10µg)	Ciprofloxacin(5µg)	Netilmicin(30µg)	IMP	IMP+EDTA	CAZ	CAZ+EDTA		IMP+EDTA	CAZ+EDTA	IMP+EDTA	CAZ+EDTA		
37	892	55/F	Burns	WS	R	R	S	S	S	S	S	S	R	S	S	S	0.25		2								
38	6628	8/Mch	UTI	Urine	R	R	S	S	S	S	S	S	S	S	S	S	0.25		1								
39	946	28/F	Burns	WS	R	R	R	R	R	R	S	R	R	R	R	R	256	2	1024	16	N	P	P	P	P	P	I
40	6574	3/Fch	DKA	Urine	R	S	S	S	S	S	S	S	S	S	S	S	1		8								
41	6974	74/F	pyuria	Urine	R	R	S	S	S	S	S	S	R	S	S	S	0.25		2								
42	1512	54/M	COPD	Sputum	R	S	S	S	S	S	S	S	S	S	S	S	0.25		8								
43	137	1/365/Mch	Sepsis	Blood	S	S	S	S	S	S	S	S	R	S	R	S	1		1								
44	187	23/F	UTI	Urine	S	S	S	S	S	S	S	S	R	S	R	S	4		0.5								
45	6211	52/F	DM foot	Pus	S	S	S	S	S	S	S	S	R	R	R	S	2		0.5								
46	6032	16/M	RTA,VAP	ET asp	S	S	S	S	S	S	S	S	R	R	R	S	0.25		1								
47	543	25/365Mch	Sepsis	Blood	S	S	S	S	S	S	S	S	S	R	R	S	4		4								
48	548	43/M	Sepsis	Blood	R	R	S	S	S	S	S	S	R	R	R	S	0.25		2								
49	734	7/Mch	UTI	Urine	S	S	S	S	S	S	S	S	S	R	R	S	0.25		1								
50	923	18/F	Burns	WS	R	R	R	R	R	R	S	R	R	R	R	R	32	2	128	8	N	P	N	P	P	P	N
51	702	30/F	Burns	WS	S	S	S	S	S	S	S	S	R	R	R	S	0.5		1								
52	918	8/Fch	UTI	Urine	R	S	S	S	S	S	S	S	S	R	R	S	1		4								
53	1140	27/M	UTI	Urine	R	R	S	S	S	S	S	S	R	R	R	S	1		4								
54	63	50/F	Pne	BAL	R	R	S	S	S	S	S	S	S	R	R	S	2		2								
55	401	43/M	COPD	Sputum	R	S	S	S	S	S	S	S	R	R	R	S	1		8								
56	1330	45/M	pyelo	Urine	R	S	S	S	S	S	S	S	R	R	R	S	4		4								

S.NO	MICRO NO	AGE/SEX	DIAGNOSIS	SPECIMEN	ANTIBIOGRAM												AGAR DILUTION (MIC)				MHT	DDST		CDDT		E TEST	PCR (IMP/VIM GENE)
					Piperacillin(75µg)	Piperacillin-tazobactam(100/10µg)	Ceftazidime(30µg)	Ceftriaxone(30µg)	Imipenem(10µg)	Meropenem(10µg)	Polymyxin B(300U)	Gentamicin(10µg)	Amikacin(30µg)	Norfloxacin(10µg)	Ciprofloxacin(5µg)	Netilmicin(30µg)	IMP	IMP+EDTA	CAZ	CAZ+EDTA		IMP+EDTA	CAZ+EDTA	IMP+EDTA	CAZ+EDTA		
57	12	45/M	VAP	ET asp	R	R	S	S	S	S	S	S	S	R	R	S	1		2								
58	778	43/M	O.E	Pus	R	R	S	S	S	S	S	R	R	R	R	S	1		2								
59	632	23/F	Burns	WS	R	R	S	S	S	S	S	S	R	R	R	S	1		2								
60	920	40/F	Burns	WS	R	R	R	R	R	R	S	R	R	R	R	R	32	2	256	8	P	P	P	P	P	P	I
61	1742	48/F	Sepsis	Blood	R	R	S	S	S	S	S	S	R	R	R	S	1		4								
62	712	72/M	Pyothorax	Pus	R	R	S	S	S	S	S	S	R	R	R	S	0.5		48								
63	2155	51/M	UTI	Urine	R	R	S	S	S	S	S	S	R	R	R	S	1		0.5								
64	13	35/F	Ascites	Asc fluid	R	S	S	S	S	S	S	S	R	R	R	S	4		0.5								
65	918	60/F	Burns	WS	R	S	S	S	S	S	S	S	R	R	R	S	2		0.5								
66	6	30/M	Abs. L thigh	Pus	R	R	S	S	S	S	S	S	R	R	R	S	2		2								
67	812	45/M	om	Pus	S	S	S	S	S	S	S	S	R	S	R	S	2		4								
68	805	52/M	DM foot	Pus	S	S	S	S	S	S	S	S	R	R	R	S	4		1								
69	814	65/M	Abscess	Pus	S	S	S	S	S	S	S	S	R	S	R	S	2		0.5								
70	678	65/M	OE	Pus	S	S	S	S	S	S	S	S	R	R	R	S	4		1								
71	221	47/M	SSI	Pus	R	R	S	S	S	S	S	S	R	R	R	R	1		4								
72	1132	28/M	csom	Pus	R	R	S	S	S	S	S	S	R	R	R	R	2		1								
73	1128	40/M	DM foot	Pus	R	R	R	R	R	R	S	S	S	R	R	R	16	0.5	128	8	P	P	P	P	P	P	I
74	608	50/M	Burns	WS	R	R	S	S	S	S	S	R	R	R	R	R	2		4								
75	1154	25/F	Pyothorax	Pus	R	R	R	R	R	R	S	R	R	R	R	R	0.5		0.5								
76	1143	30/M	crush injury	Pus	R	R	R	R	R	R	S	R	R	R	R	R	1		4								

S.NO	MICRO NO	AGE/SEX	DIAGNOSIS	SPECIMEN	ANTIBIOGRAM												AGAR DILUTION (MIC)				MHT	DDST		CDDT		E TEST	PCR (IMP/VIM GENE)
					Piperacillin(75µg)	Piperacillin-tazobactam(100/10µg)	Ceftazidime(30µg)	Ceftriaxone(30µg)	Imipenem(10µg)	Meropenem(10µg)	PolymyxinB(300U)	Gentamicin(10µg)	Amikacin(30µg)	Norfloxacin(10µg)	Ciprofloxacin(5µg)	Netilmicin(30µg)	IMP	IMP+EDTA	CAZ	CAZ+EDTA		IMP+EDTA	CAZ+EDTA	IMP+EDTA	CAZ+EDTA		
77	1034	15/F	Burns	WS	R	R	R	R	S	S	S	R	R	R	R	R	0.25	-	256	128	N	N	P	N	P	N	I
78	1233	50/F	DM cellulitis	Pus	R	R	S	S	S	S	S	R	R	R	R	R	1		4								
79	1208	40/M	Malignancy,SSI	Pus	R	R	R	R	R	R	S	R	R	R	R	R	32	2	256	8	P	P	P	P	N	P	N
80	1204	50/F	Burns	Pus	R	R	S	S	S	S	S	R	R	R	R	R	4		8								
81	1017	48/F	DMfoot	Pus	R	R	S	S	S	S	S	R	R	R	R	R	1		0.5								
82	644	50/M	Burns	WS	R	R	R	R	R	R	S	R	R	R	R	R	32	1	512	8	P	P	P	P	P	P	I
83	159	18/M	OPC , VAP	ET Asp	R	R	R	R	R	R	S	R	S	R	S	R	32	0.5	512	8	P	P	P	N	P	P	I
84	3464	12/M	UTI	Urine	S	S	S	S	S	S	S	R	R	R	R	R	1		4								
85	1319	32/M	RTI,VAP	Sputum	S	S	S	S	S	S	S	R	R	R	R	R	0.25		1								
86	6144	63/F	DM foot	Pus	S	S	S	S	S	S	S	R	R	R	R	R	1		2								
87	3825	5/Mch	UTI	Urine	S	S	S	S	S	S	S	R	R	R	R	R	1		1								
88	896	35/F	Burns	WS	S	S	R	R	S	S	S	R	R	R	R	R	1		1								
89	916	50/M	Burns	WS	S	S	S	S	S	S	S	R	R	R	R	R	1		0.5								
90	1170	70/F	Pneumonia	Sputum	R	R	S	S	S	S	S	R	R	R	R	R	1		8								
91	182	45/F	Empyema	Pus	R	R	S	S	S	S	S	R	R	R	R	R	1		4								
92	1189	55/F	Pneumonia	Sputum	R	R	R	R	S	S	S	R	S	R	R	R	0.25	_	64	2	N	N	P	N	P	P	I
93	1756	55/M	COPD	Sputum	R	R	S	S	S	S		R	R	R	R	R	1		2								
94	1508	35/F	SSI	Pus	R	R	R	R	S	S	S	R	S	R	R	R	16	0.25	256	4	N	P	P	P	P	P	I
95	4078	56/M	Crush injury,Prolonged use of antibiotic	WS	R	R	R	R	R	R	S	R	S	R	R	R	16	0.5	256	8	P	P	P	P	P	P	I

S.NO	MICRO NO	AGE/SEX	DIAGNOSIS	SPECIMEN	ANTIBIOGRAM												AGAR DILUTION (MIC)				MHT	DDST		CDDT		E TEST	PCR (IMP/VIM GENE)
					Piperacillin(75µg)	Piperacillin-tazobactam(100/10µg)	Ceftazidime(30µg)	Ceftriaxone(30µg)	Imipenem(10µg)	Meropenem(10µg)	PolymyxinB(300U)	Gentamicin(10µg)	Amikacin(30µg)	Norfloxacin(10µg)	Ciprofloxacin(5µg)	Netilmicin(30µg)	IMP	IMP+EDTA	CAZ	CAZ+EDTA		IMP+EDTA	CAZ+EDTA	IMP+EDTA	CAZ+EDTA		
96	906	25/F	Burns	WS	S	S	S	S	S	S	S	R	R	R	R	R	1		4								
97	1206	9/365/Fch	Pneumonia	BAL	R	R	R	R	S	S	S	R	R	R	R	R	16	1	128	4	N	P	P	P	P	P	I
98	1209	24/F	CRF	Urine	R	R	R	R	S	S	S	IS	R	s	R	R	16	0.5	32	1	N	P	P	P	P	P	I
99	1341	13/M	CCF	Sputum	R	R	S	S	S		S	R	R	R	R	R	1		8								
100	907	32/M	Burns	WS	R	R	R	R	R	R	S	R	R	R	R	R	32	1	256	8	P	P	P	P	P	P	I
101	1339	60/F	Pneumonia	Sputum	S	S	S	S	S	S	S	R	R	R	R	R	1		8								
102	1346	55/M	empyema	Sputum	R	R	S	S	R	R	S	R	R	R	R	R	1		2								
103	1125	50/M	UL cons	Sputum	R	R	S	S	S	S	S	R	R	R	R	R	1		4								
104	910	31/M	Burns	WS	R	R	S	S	S	S	S	R	R	R	R	R	1		4								
105	913	27/F	Burns	WS	R	S	S	S	R	R	S	R	R	R	R	R	1		8								
106	4848	3/Mch	CP,MR,UTI	Urine	R	R	R	R	R	R	S	R	R	R	R	R	16	0.5	1024	8	N	P	P	P	P	P	I +V
107	914	37/M	Burns	WS	R	R	R	R	R	R	S	R	R	R	R	R	32	2	256	16	P	P	P	P	P	P	I
108	927	65/M	Pneumonia	Sputum	R	R	R	R	R	R	S	R	R	R	R	R	32	2	256	8	P	P	P	P	P	P	I

Pne-Pneumonia

WI-wound infection

WS-wound swab

CA rect - Carcinoma rectum

SSI - Surgicial Site Infection

O.E - Otitis Externa

pyelo - pyelo nephritis

DM - Diabetes Mellitus

I-IMP gene

V-VIM gene

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INTRODUCTION Pseudomonas aeruginosa has become one of the most dreadful causes of nosocomial bacterial infections especially in the lung, blood and urinary tract. As a result of its considerable potential to become resistant to many antibiotics more multidrug resistant strains are encountered as clinical isolates, leaving physicians with a decreasing armamentarium of effective drugs for treatment. Before the advent of modern medical microbiology, there was evidence that P.aeruginosa was a cause of serious wound and surgical infections, as elaborated by Doggett .In 1850, it was noted by Sedillot that there were sometimes blue green discharges on surgical dressings that were associated with...

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